The genetic analyses of DNA repair pathway genes in French Canadians of Quebec identified new candidate risk variants implicated in hereditary ovarian cancer

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DEDICATION

I dedicate this thesis to my beloved mom, Amaal, and my siblings, Afnan, Yazid and Abdul-Rahman, for their endless support during my journey

I also dedicate this thesis to all women affected with ovarian cancer and to their family members for their contributions to research

ABSTRACT

Rare germline pathogenic variants (PVs) in clinically actionable ovarian cancer predisposing genes (OCPGs) such as *BRCA1* and *BRCA2*, involved in the homologous recombination (HR) pathway, and *MLH1, MSH2, MSH6 and PMS2*, involved in the mismatch repair (MMR) pathway, do not account for all hereditary ovarian cancer (OC) cases, including those associated with familial cancer. This led to my hypothesis that there are other OC risk variants/genes to be discovered. However, there is a growing consensus that the carrier frequencies of variants in these new risk genes are less common than for the established OCPGs, which has created challenges in their discovery. To overcome these challenges, I proposed to apply whole exome sequencing (WES) and bioinformatic analyses to identify candidate OC risk variants by investigating: (1) OC families and sporadic cases as well as controls from the French Canadian (FC) population of Quebec as this population is known to exhibit founder effects due to common ancestors; (2) *BRCA1* and *BRCA2* PV-negative families with at least two close relatives with OC and/or sporadic early-onset cases (diagnosed with OC before the age of 50 years) who were not selected for family history of cancer as both of these groups represent phenotypic hallmarks of genetic predisposition; and (3) rare germline variants in DNA repair pathway genes using a candidate gene approach as the established and known OCPGs have been shown to play a role in these pathways. I applied these three strategies to execute four main aims to test my hypothesis. For aim 1 (Chapter II), I identified a rare intronic likely pathogenic variant (LPV) *BRCA1* c.5407-25T>A in one of the 22 OC families and one of the 53 early-onset OC cases who had previously tested negative for *BRCA1* and *BRCA2* PVs in a medical genetic setting. For aim 2 (Chapter III), I identified five candidate variants in known OC risk genes: *RAD51C* and *RAD51D,* in 18% of the 17 FC families and 11% of the 53 early-onset cases; validated the high carrier frequency of the LPV *RAD51D* c.620C>T; p.Ser207Leu in OC families and sporadic cases; identified multiple OC carriers of a LPV *RAD51C* c.705G>T; p.Lys235Asn; and identified three other rarer PVs or LPVs in *RAD51C* or *RAD51D*. For aim 3 (Chapter IV), I identified five candidate variants in another known OC risk gene *BRIP1* in FC cancer cases that were initially reported in cancer cases including OC by adult hereditary cancer clinics in Quebec; and identified multiple OC carriers of LPVs

c.797C>T; p.Thr266Met; c.2087C>T; p.Pro696Leu and c.2990_2993delCAAA; p.Thr997ArgfsTer61. For the last aim (Chapter V), I identified a new candidate OC risk variant in each of five genes involved in various DNA repair pathways in 39% of the 13 FC OC families: *ERCC5* (nucleotide excision repair [NER] and base excision repair [BER] pathways), *EXO1* (HR and MMR pathways), *FANCC* (Fanconi anemia [FA] pathway), *NTHL1* and *NEIL1* (BER pathway); and showed low carrier frequencies in 435 sporadic OC cases: 0.5% for each of the three variants *ERCC5* c.2556A>G; p.Ile852Met, *NEIL1* c.248G>T; p.Gly83Asp and *NTHL1* c.244C>T; p.Gln82Ter, 0.2% for of *EXO1* c.1268-1G>T and none for *FANCC* c.897G>T; p.Arg299Ser. In conclusion, my strategy of applying WES and bioinformatics analyses combined with a candidate gene approach focusing on genes involved in DNA repair pathways to the germline of OC families and cases from a population exhibiting founder effect and genetic drift successfully identified: (1) new PVs or LPVs in known OC risk genes *RAD51C, RAD51D* and *BRIP1*, supporting their role in OC risk in the FC population; and (2) new candidates in new OC risk genes *ERCC5*, *EXO1*, *FANCC*, *NEIL1* and *NTHL1*, supporting my hypothesis that there are other DNA repair pathway genes implicated in predisposition to OC.

RÉSUMÉ

Les variants pathogéniques (VP) germinaux rares présents dans les gènes de prédisposition au cancer de l'ovaire (GPCO) cliniquement exploitables, tels que *BRCA1* et *BRCA2*, impliqués dans la voie de la recombinaison homologue (HR), et *MLH1*, *MSH2*, *MSH6* et *PMS2*, impliqués dans la voie de réparation des mésappariements (MMR), n'expliquent pas tous les cas de cancer de l'ovaire (CO) héréditaires, incluant ceux associés au cancer familial. J'ai donc émis l'hypothèse qu'il existait d'autres gènes ou variants de risque de CO à découvrir. Cependant, il y a un consensus grandissant selon lequel les fréquences de porteurs de variants dans ces nouveaux gènes de risque sont plus rares que pour les GPCO établis, ce qui a créé des difficultés pour leur découverte. Pour surmonter ces défis, j'ai proposé d'appliquer le séquençage de l'exome entier (WES) et des analyses bioinformatiques pour identifier des candidats variants de risque de CO en étudiant: (1) des familles et des cas sporadiques de CO ainsi que des contrôles dans la population canadienne française (CF) du Québec, car cette population est connue pour présenter des effets fondateurs dus à des ancêtres communs; (2) des familles *BRCA1* et *BRCA2* négatives pour des VP avec au moins deux parents proches atteints de CO et/ou des cas sporadiques diagnostiqués précocement (avant l'âge de 50 ans) du CO et qui n'ont pas été sélectionnés pour leurs antécédents familiaux de cancer, car ces deux groupes présentent les caractéristiques phénotypiques de la prédisposition génétique; et (3) les variants germinaux rares dans les gènes de la voie de réparation de l'ADN en utilisant une approche de gène candidat, car il a été démontré que les GPCO établis et connus jouent un rôle dans ces voies. J'ai appliqué ces trois stratégies pour atteindre mes quatre objectifs principaux et tester mon hypothèse. Pour l'objectif 1 (Chapitre II), j'ai identifié un variant intronique rare probablement pathogène (VPP) *BRCA1* c.5407-25T>A dans l'une des 22 familles de CO et l'un des 53 cas précoces de CO testés précédemment négatifs pour les VP de *BRCA1* et *BRCA2* dans le cadre d'un test en génétique médicale. Pour l'objectif 2 (Chapitre III), j'ai identifié cinq candidats variants dans des gènes de risque de CO connus: *RAD51C* et *RAD51D*, dans 18% des 17 CF familles et 11% des 53 cas précoces; j'ai validé la fréquence élevée de porteurs du VPP *RAD51D* c.620C>T; p.Ser207Leu dans les familles de CO et les cas sporadiques; j'ai identifié de multiples

cas de CO porteurs d'un VPP *RAD51C* c.705G>T; p.Lys235Asn; et j'ai identifié trois autres VP ou VPP plus rares dans *RAD51C* ou *RAD51D*. Pour le troisième objectif (Chapitre IV), j'ai identifié cinq candidats variants dans un autre gène connu de risque de CO, *BRIP1*, dans des cas CF de cancer, qui avaient été rapportés initialement par des cliniques de cancer héréditaire pour adultes au Québec dans des cas de cancer, dont CO; et j'ai identifié de multiples cas de CO porteurs des VPP c.797C>T; p.Thr266Met; c.2087C>T; p.Pro696Leu et c.2990_2993delCAAA; p.Thr997ArgfsTer61. Pour le dernier objectif (Chapitre V), j'ai identifié un nouveau candidat variant de risque de CO dans chacun des cinq gènes impliqués dans diverses voies de réparation de l'ADN dans 39% des 13 CF familles de CO: *ERCC5* (voies de réparation par excision des nucléotides [NER] et réparation par excision des bases [BER]), *EXO1* (voies HR et MMR), *FANCC* (voie de l'anémie de Fanconi [FA]), *NTHL1* et *NEIL1* (voie BER); et j'ai montré une faible fréquence de porteurs dans 435 cas de CO sporadiques: 0,5% pour chacun des trois variants *ERCC5* c.2556A>G; p.Ile852Met, *NEIL1* c.248G>T; p.Gly83Asp et *NTHL1* c.244C>T; p.Gln82Ter, 0,2% pour *EXO1* c.1268-1G>T et aucun pour *FANCC* c.897 G>T; p.Arg299Ser. En conclusion, ma stratégie consistant à appliquer des analyses WES et bioinformatiques combinées à une approche des gènes candidats axée sur les gènes impliqués dans les voies de réparation de l'ADN à des familles la lignée germinale et à des cas de CO provenant d'une population présentant un effet fondateur et une dérive génétique a permis d'identifier (1) de nouveaux VP ou VPP dans les gènes de risque de CO connus *RAD51C*, *RAD51D* et *BRIP1*, soutenant leur rôle dans le risque de CO dans la population CF; et (2) de nouveaux candidats dans les nouveaux gènes de risque de CO *ERCC5*, *EXO1*, *FANCC*, *NEIL1* et *NTHL1*, soutenant mon hypothèse selon laquelle il existe d'autres gènes des voies de réparation de l'ADN impliqués dans la prédisposition au CO.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
ACMG	The American College of Medical Genetics and Genomics guidelines
AD	Autosomal Dominant
ADA	AdaBoost prediction in silico tool
AR	Autosomal Recessive
AUS	Cases from the AUStralian population
BAM	Binary Version of Raw Sequencing Data
BC	Breast Cancer
BCDX2	RAD51B-RAD51C-RAD51D-XRCC2 complex
BER	Base Excision Repair
bp	Base Pair
BP1	Benign Supporting Level 1 variant classification
BP6	Benign Supporting Level 6 variant classification
BRCT	BRCA1 Carboxy Terminus domain
BS1	Benign Strong Level 1 variant classification
BSA	Bovine Serum Albumin
BSO	Bilateral Salpingo-Oophorectomy
CA125	Cancer Antigen 125
CADD	Combined Annotation Dependent Depletion prediction in silico tool
CCC	Clear Cell Ovarian Carcinoma
CHX	Cycloheximide
CI	Confidence Interval
CIC	Cortical Inclusion Cysts
cM	Centimorgan
CNV	Copy Number Variant
CONDEL	CONsensus DELeteriousness prediction in silico tool
COSMIC	Catalogue Of Somatic Mutations In Cancer
CPG	Cancer Predisposing Gene
CSV	Comma Separated Values files
CTL	Control, unspecific

CX3	RAD51C-XRCC3 complex
DAPI	4,6-diamidino- 2-phenylindole
dbscSNV	Database Splicing Consensus Single Nucleotide Variant
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EC	Endometrioid Ovarian Carcinoma
EV	Empty Vector
FA	Fanconi Anemia
FBS	Fetal Bovine Serum
FC	French Canadian
Fe-S	Iron-Sulfur Domain
FF	Fresh-Frozen tumour DNA
FFPE	Formalin-Fixed Paraffin-Embedded tumour DNA
FIGO	International Federation of Gynecology and Obstetrics
FLOSSIES	Fabulous Ladies Over Seventy Database
GATK	Genome Analysis Tool Kit
Gen3G	The Genetics of Glucose Regulation in Gestation and Growth Biobank
GERP	Genomic Evolutionary Rate Profiling prediction in silico tool
gnomAD	The genome Aggregation Database
GWAS	Genome-Wide Association Studies
HBC	Hereditary Breast Cancer
HBOC	Hereditary Breast and Ovarian Cancer
HGSC	High-Grade Serous Ovarian Carcinoma
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HR	Hazard Ratio
HR	Homologous Recombination
HRC	Haplotype Reference Consortium
HSF	Human Splicing Finder prediction in silico tool
ICL	Inter- or Intra-Strand CrossLinks
iCOGS	Collaborative Oncological Gene-Environment Study
IGV	Integrative Genomics Viewer Software

KO	Knock-Out
LCL	Lymphoblastoid-derived Cell Line
LGSC	Low-Grade Serous Ovarian Carcinoma
LOF	Loss-of-Function
Log	Logarithm
LOH	Loss of Heterozygosity
LPV	Likely Pathogenic Variant
LS	Lynch Syndrome
MAF	Minor Allele Frequency
MaxEntScan	Maximum Entropy Estimates of Splice Junction prediction in silico tool
Mb	Megabase pair
MBD	MLH1 Binding domain
MC	Mucinous Ovarian Carcinoma
MetaLR	Meta-analytic Logistic Regression prediction in silico tool
MetaSVM	Meta-analytic support Vector Machine
MINAS	Multi-Locus Inherited Neoplasia Allele Syndrome
MIX	Cases of MIXed ethnicity
MMC	Mitomycin C
MMR	Mismatch Repair
MNI	Montreal Neurological Institute Biobank
MSI	Microsatellite Instability
NCBI	National Center for Biotechnology Information
NCCN	National Comprehensive Cancer Network guidelines for clinical practice
NER	Nucleotide Excision Repair
OC	Ovarian Cancer
OCAC	Ovarian Cancer Association Consortium database
OCP	Oral Contraceptives
OCPG	Ovarian Cancer Predisposing Gene
OMIM	Online Mendelian Inheritance in Man database
OR	Odds Ratio
PARP	Poly (ADP-Ribose) Polymerase

PBL	Peripheral Blood Lymphocytes
PBS	Phosphate Buffered Saline
PhastCons	PHAST Conservation of 100 vertebrates prediction in silico tool
PhyloP	Phylogenetic P value of 100 vertebrates prediction in silico tool
PM2	Pathogenic Moderate Level 2
PP3	Pathogenic Supporting Level 3
PP5	Pathogenic Supporting Level 5
PROVEAN	Protein Variation Effect Analyzer prediction in silico tool
PRS	Polygenic Risk Score
PS1	Pathogenic Strong Level 1
PS3	Pathogenic Strong Level 3
PV	Pathogenic Variants
PVS1	Pathogenic Very Strong Level 1
REVEL	Rare Exome Variant Ensemble Learner prediction in silico tool
RF	Random Forest prediction in silico tool
RR	Relative Risk
RRM	Risk-Reducing Mastectomy
RRSO	Risk-Reducing Salpingo-Oophorectomy
RT-PCR	Real Time Polymerase Chain Reaction
SE	Standard Error
SEM	Standard Error of the Mean
SIR	Standardised Incidence Ratio
SNP	Single Nucleotide Polymorphism
STIC	Serous Tubal Intraepithelial Carcinoma
TCGA	The Cancer Genome Atlas
VAF	Variant Allele Frequency
VCF	Variant Call Format
VEP	Variant Effect Predictor
VEST	Variant Effect Scoring Test prediction in silico tool
VUS	Variant of Uncertain Significance
WES	Whole Exome Sequencing

WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild-Type

All genes mentioned in this thesis are referred to by the official gene symbol based on The National Center for Biotechnology Information (NCBI) – Gene Database (ncbi.nlm.nih.gov) and their annotations using the canonical transcripts are based on (tark.ensemble.org); all can be found in **Appendix VIII**.

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THESIS FORMAT

This doctoral thesis is written in a manuscript-based format which consists of seven chapters. **Chapter I** is an introduction where I provided a literature overview focused on the epidemiology, the etiology and the histopathological subtypes of ovarian cancer and the current standard of care of ovarian cancer followed by an in-depth review of hereditary ovarian cancer and associated epidemiological and heritable genetic risk variants/genes. In the last section of this chapter, I presented the missing heritability of ovarian cancer and the possible challenges in discovering new genetic risk variants/genes for ovarian cancer. This followed by presenting the rationale of this thesis project and my hypothesis. Lastly, I presented specific strategies to be applied by executing four main aims to test my hypothesis. **Chapters II**, **III**, **IV** and **V** are manuscripts of four independent original research articles where I am the first author, and each corresponding to one of the four main aims; two of which have been published. **Chapter VI** is a general discussion where I summarized the main findings of my research articles (Chapters II to V) and discussed their contributions to the current knowledge, potential limitations and possible future directions, and **Chapter VII** is an overall conclusion.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The work presented in this thesis constitutes an original contribution to the identification and characterization of rare germline candidate risk variants in known or new ovarian cancer predisposing genes. The results presented in this thesis were derived from whole exome sequencing and bioinformatics analyses in combination with my proposed strategies of applying a candidate gene approach involving known or putative genes in DNA repair pathways; and investigating selected familial and sporadic ovarian cancer cases from the French Canadians of Quebec, a population exhibiting a unique genetic architecture due to founder effects. I identified: (1) pathogenic or potentially deleterious variants in known ovarian cancer risk genes, *BRCA1*, *RAD51C*, *RAD51D* and *BRIP1*; and (2) potentially deleterious candidate variants in new genes that have never been associated with hereditary ovarian cancer, *ERCC5*, *EXO1*, *FANCC*, *NEIL1* and *NTHL1*. I provided further evidence by targeted genotyping in multiple cancer study groups of French Canadians and by genetic analyses of ovarian tumour tissues from the ovarian cancer carriers of the candidate variants. Collectively, my analyses supported the potential pathogenic role of the identified candidate variants in known and new genes implicated in ovarian cancer risk using specific strategies in the population of French Canadians, which may also be an advantage for non-French Canadian populations.

CONTRIBUTION OF AUTHORS

The original research studies presented in this thesis were designed, conducted, analyzed and written during the past years by me, Wejdan M. Alenezi, as a PhD student in the Department of Human Genetics at McGill University and under the supervision of Dr. Patricia Tonin.

CHAPTER I: Introduction

I reviewed and summarized peer-reviewed original articles and reviews that were related to the main focus of this thesis in order to provide the current state of knowledge in the field of genetic risk of hereditary ovarian cancer, and to frame my hypothesis and main aims. This was conducted under the guidance of Dr. Patricia Tonin.

CHAPTER II: Case Review: Whole-Exome Sequencing Analyses Identify Carriers of a Known Likely Pathogenic Intronic *BRCA1* Variant in Ovarian Cancer Cases Clinically Negative for Pathogenic *BRCA1* and *BRCA2* Variants

Wejdan M. Alenezi, Caitlin T. Fierheller, Timothée Revil, Corinne Serruya, Anne-Marie Mes-Masson, William D Foulkes, Diane Provencher, Zaki El Haffaf, Jiannis Ragoussis and Patricia N. Tonin.

I conceptualized and designed the study under the supervision of Dr. Patricia Tonin, performed whole exome sequencing and bioinformatics analyses, performed the required molecular genetics analyses, wrote the first draft of the manuscript and edited and reviewed the final draft of the manuscript. Dr. Timothée Revil performed the whole exome sequencing pipeline for germline variant calling under the supervision of Dr. Jiannis Ragoussis. Caitlin T. Fierheller aided in the molecular genetics analyses, and Corinne Serruya aided in collecting and reviewing the clinico-pathological and genetic data from the adult hereditary cancer clinics. Drs. Anne-Marie Mes-Masson, William D Foulkes, Diane Provencher and Zaki El Haffaf provided DNA samples and genetic and clinico-pathological data of cancer cases. All authors have read and approved the submitted version of the manuscript.

CHAPTER III: The Genetic and Molecular Analyses of *RAD51C* and *RAD51D* Identifies Rare Variants Implicated in Hereditary Ovarian Cancer from a Genetically Unique Population

Wejdan M. Alenezi, Larissa Milano, Caitlin T. Fierheller, Corinne Serruya, Timothée Revil, Kathleen K. Oros, Supriya Behl, Suzanna L. Arcand, Porangana Nayar, Dan Spiegelman, Simon Gravel, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau, Luigi Bouchard, Celia M.T. Greenwood, Jean-Yves Masson, Jiannis Ragoussis and Patricia N. Tonin

I conceptualized and designed the study under the supervision of Dr. Patricia Tonin, performed whole exome sequencing and bioinformatics analyses, performed targeted genotyping and other molecular genetics analyses, performed statistical analyses, wrote the first draft of the manuscript and edited and reviewed the final draft of the manuscript. Dr. Larissa Milano performed the in cellulo assays under the supervision of Dr. Jean-Yves Masson. Corinne Serruya aided in collecting and reviewing the clinico-pathological and genetic data from the adult hereditary cancer clinics. Dr. Timothée Revil performed the whole exome sequencing pipeline for germline variant calling under the supervision of Dr. Jiannis Ragoussis. Dr. Kathleen K. Oros performed imputation of genotyping data under the supervision of Dr. Celia M.T. Greenwood. Caitlin T. Fierheller, Supriya Behl, Suzanna L. Arcand and Porangana Nayar aided in some of the molecular genetics analyses under the supervision of Drs. William D. Foulkes or Patricia Tonin. Dan Spiegelman and Drs. Simon Gravel, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau and Luigi Bouchard provided DNA samples and genetic and/or clinico-pathological data of cancer cases and controls. All authors have read and approved the submitted version of the manuscript.

CHAPTER IV: Genetic and Molecular Analyses of Candidate *BRIP1/FANCJ* Variants Implicated in Breast and Ovarian Cancers

Larissa Milano†, Wejdan M. Alenezi†, Caitlin T. Fierheller, Corinne Serruya, Timothée Revil, Kathleen K. Oros, Jeffrey Bruce, Dan Spiegelman, Simon Gravel, Trevor Pugh, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy

Rouleau, Luigi Bouchard, Celia M.T. Greenwood, Jiannis Ragoussis, Patricia N. Tonin and Jean-Yves Masson

† Contributed equally to this study as co-first authors.

I conceptualized and designed the study under the supervision of Dr. Patricia Tonin, performed whole exome sequencing and bioinformatics analyses, performed targeted genotyping and the other required molecular genetics analyses, performed required statistical analyses, wrote the first draft of the manuscript and edited and reviewed the final draft of the manuscript. Dr. Larissa Milano performed the in cellulo assays under the supervision of Dr. Jean-Yves Masson. Caitlin T. Fierheller aided with the bioinformatics analyses, and Corinne Serruya aided in collecting and reviewing the clinico-pathological and genetic data from the adult hereditary cancer clinics. Dr. Timothée Revil performed the whole exome sequencing pipeline for germline variant calling under the supervision of Dr. Jiannis Ragoussis. Dr. Kathleen K. Oros performed imputation of genotyping data under the supervision of Dr. Celia M.T. Greenwood. Dan Spiegelman and Drs. Jeffrey Bruce, Simon Gravel, Trevor Pugh, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau and Luigi Bouchard provided DNA samples and genetic and/or clinico-pathological data of cancer cases and controls. All authors have read and approved the revised manuscript for submission.

CHAPTER V: Genetic Analyses of DNA Repair Pathway Associated Genes Implicates New Candidate Cancer Predisposing Genes in Ancestrally Defined familial and sporadic Ovarian Cancer Cases

Wejdan M. Alenezi, Caitlin T. Fierheller, Corinne Serruya, Timothée Revil, Kathleen K. Oros, Deepak N. Subramanian, Jeffrey Bruce, Dan Spiegelman, Simon Gravel, Trevor Pugh, Ian G. Campbell, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau, Luigi Bouchard, Celia M.T. Greenwood, Jiannis Ragoussis and Patricia N. Tonin

I conceptualized and designed the study under the supervision of Dr. Patricia Tonin, performed whole exome sequencing and bioinformatics analyses, performed targeted genotyping and the other required molecular genetics analyses, performed required

statistical analyses, wrote the first draft of the manuscript and edited and reviewed the final draft of the manuscript. Caitlin T. Fierheller aided with the bioinformatics analyses, and Corinne Serruya aided in collecting and reviewing the clinico-pathological and genetic data from the adult hereditary cancer clinics. Dr. Timothée Revil performed the whole exome sequencing pipeline for germline variant calling under the supervision of Dr. Jiannis Ragoussis. Dr. Kathleen K. Oros performed imputation of genotyping data under the supervision of Dr. Celia M.T. Greenwood. Dan Spiegelman and Drs. Deepak N. Subramanian, Jeffrey Bruce, Dan Spiegelman, Simon Gravel, Trevor Pugh, Ian G. Campbell, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau and Luigi Bouchard provided DNA samples and genetic and/or clinico-pathological data of cancer cases and controls. All authors have read and approved the revised manuscript for submission.

CHAPTER VI: General discussion

I wrote the general discussion of this thesis to summarize the overall results in Chapters II to V and discussed how these findings provided insight into the current knowledge of the field of genetic risk for hereditary ovarian cancer. I also discussed the main limitations of the original research study designs and possible future directions that could be conducted in both the short and long term.

CHAPTER VII: Conclusion

I provided an overall conclusion of the results presented in Chapters II to V.

CHAPTER I: Introduction

1.1. Epidemiology of ovarian cancer

Ovarian cancer (OC) is the tenth most prevalent cancer among women in Canada [1]. The lifetime probability for developing the disease is estimated to be 1 in 79 Canadian women [1]. The age-adjusted incidence rate is 13.4 per 100,000 OC women in Canada, which is comparable in all Canadian provinces and territories [1]. Approximately 3,000 new cases are estimated in 2022 [2]. On a global scale, OC is ranked the seventh to eighth on the list of the most prevalent cancers among women, and approximately 1 in 100 women worldwide is expected to be diagnosed with OC during their lifetime [3]. The incidence rate of OC varies across the world where the range of age-adjusted incidence rates is between 3.0 and 11.4 per 100,000 women with the highest rates observed in Central and Eastern European countries [3–10].

The Canadian Cancer Statistics 2021 reported that there has been an overall decrease in the OC incidence in Canada and some other countries [1]. It has been reported that the incidence rate has declined by approximately -1.5% (95% confidence interval [CI]: -1.9 to -1.0) between 1984 and 1997, and since then it levelled off until 2013 [1]. Since then and until 2017, there has been a rapid drop in the incidence rate by approximately -3.1% (95% CI: -5.3 to -0.8) per year [1]. Overall, the OC incidence in Canada is 27% lower in 2021 than in 1984 [1]. A similar trend has also been observed in the United States and other countries in Western Europe [1], although the incidence rate continues to increase in other countries in Central and Eastern Europe and Asia [9,10]. This trend can be explained by the use of oral contraceptives (OCPs) as a primary protective risk factor for OC (presented in **section 1.5.1.2.**) which its use has increased in North America and Western Europe [10,11]. Another factor that could explain the decreased incidence trend of OC is excluding any form of ovarian neoplasm such as benign or borderline epithelial tumours of the ovary, which collectively account for approximately 15% of all OC cases, from the annual estimation of OC incidence rate [1]. This has occurred based on the updated guidelines for OC subtypes classification by the World Health Organization (WHO) in 2000 [10,12], revised in 2014 [13–15] and remain valid since then [10,16].

In spite of the favourable trend in the disease incidence, OC remains the leading cause of death among gynecological cancers in Canada and worldwide [3–10]. OC is the fifth cause of death among women in Canada [1]. The lifetime probability of dying from OC is estimated to be 1 in 103 Canadian women, and the age-adjusted mortality rate is 8.0 per 100,000 with 1,950 projected deaths due to OC in 2022 [1,2]. The OC mortality rate is almost uniform across all Canadian provinces and territories [1]. Worldwide, OC is the seventh to eighth leading cause of death among women, and approximately 1 in 108 women are expected to die from OC during their lifetime [3–6]. The OC mortality rate varies across the world where the age-adjusted mortality rate due to OC is 4.2 per 100,000 deaths [3–5,8]. In most European countries, the age-adjusted five-year survival rate for OC is 62.4% (95% CI: 61.8% to 62.6%) [3,9]. Whereas, this rate is approximately 44% (95% CI: 43% to 54%) in Canada [1] which is comparable to that in the United States [3,10]. These rates vary considerably with the age at diagnosis with OC and among different cancer subtypes (presented in the following **section 1.2.**).

1.2. Histopathological subtypes of ovarian cancer

OC is a heterogeneous disease [17,18]. OC is generally grouped into epithelial OC of approximately 90% and non-epithelial of the remaining cases [16,18–21]. Epithelial OC cases are fundamentally different in their potential precursor lesions (presented in the following **section 1.3.**), prognosis and response to treatment (presented in **section 1.4.**) and associated epidemiological and genetic risk factors (presented in **section 1.5.**) [15,18,22]. These tumours are graded based on the WHO guidelines into low-, medium- or high-grade tumours based on the differentiation of the cancerous cells from well-differentiated (low-grade) to poorly-differentiated (high-grade) [19–21]. These tumours are also staged based on the International Federation of Gynecology and Obstetrics (FIGO) guidelines from stage I (cancerous cells confined to either or both ovaries) to stage IV (cancerous cells spread outside the peritoneum) [13,15,21].

Epithelial OC cases comprise five distinct subtypes: high-grade serous carcinoma (HGSC), low-grade serous carcinoma (LGSC), endometrioid carcinoma (EC), clear cell carcinoma (CCC), and mucinous carcinoma (MC) [15,20,21]. The main clinico-pathological characteristics of these subtypes are summarized in **Table 1.2**. HGSC

Table 1.2. The clinico-pathological characteristics of the main epithelial ovarian cancer subtypes.

	HGSC	LGSC	EC	CCC	MC
Average age at diagnosis	Early to mid 60s	Mid 50s	Early 50s	Early 50s	Early 50s
Contribution to epithelial OC	70-75%	5%	10-12%	10-12%	3%
Contribution to each OC histopathological subtype based on the disease stage	85% of late stages III-IV	95% of early stages I-II	85-95% of early stages I-II	75% of early stages I-II	65-80% of early stages I-II
Contribution to each OC histopathological subtype based on the disease grade	90% of SC	10% of SC	5-15% of high-grade EC	10% of high-grade CCC	5% of high-grade MC
Overall five-year survival rate	15-25%	75%	80%	85-90%	90%

Percentages are in approximations and all information was summarized from references [18,20,22–31]. CCC: Clear Cell Carcinoma; EC: Endometrioid Carcinoma; HGSC: High-Grade Serous Carcinoma; LGSC: Low-Grade Serous Carcinoma; MC: Mucinous Carcinoma; and SC: Serous Carcinoma.

accounts for around three-quarters of all epithelial OC cases, and is responsible for up to 80% of all deaths from OC [32]. The overall five-year survival of HGSC is about 25%, and over 85% of those cases are diagnosed at advanced stage [15,32]. This is mainly attributed to the fact that early-stage HGSC is associated with nonspecific symptoms, such as abdominal and pelvic pain with fatigue, and those women often present in the clinic when the disease is at an advanced stage with mostly ascites and gastrointestinal dysfunction [18]. The majority of women with HGSC are diagnosed in their early to mid-60s, and about 30% are diagnosed before the age of 50 years [33,34]. HGSC tumours are characterized as solid tumours with slit-like fenestration cells combined with a necrotic component [18,25]. The cancerous cells of HGSC tumours exhibit papillary, glandular or cribriform architectures, and cytologically, high nuclear atypia, a high nuclear-to-cytoplasmic ratio [18,25]. Considerable effort has been made to differentiate HGSC cases from the other epithelial OC subtypes using a few number of immunohistochemical markers with high sensitivity and specificity such as p53 [20].

Women diagnosed with LGSC, EC, CCC or MC, on the other hand, are usually in their early mid-50s and mainly present with early stages of the disease [26,28,29,35,36]. LGSC cells also exhibit papillary architecture, but low-to-mild nuclear atypia and a low-to-mild nuclear-to-cytoplasmic ratio [18,36]. EC cells exhibit gland formation which recapitulates endometrial glands, whereas CCC tumours have large atypical cells with clear cytoplasm and stromal hyalinization-like cells [18,26]. MC cancerous cells exhibit mucin-filled cells with the presence of goblet cells [18,28].

1.3. Origin of ovarian cancer

The etiology of epithelial OC is unknown [18,37]. The fact that the histopathological characteristics of epithelial OC subtypes are different from those of normal cells of the ovary makes the process of understanding the origin of OC arduous [37]. Several hypotheses and models have been proposed to tackle the origin and pathogenesis of each subtype [18,24,37,38]. The proposed tissue and cell of origin and the possible precursor lesion of the main epithelial OC subtypes are summarized in **Table 1.3.1.** and the genomic characteristics of each of these subtypes are presented in **Table 1.3.2.** in an effort to understand their origin and pathogenesis.

Table 1.3.1. The proposed origin of ovarian cancer by subtype.

	HGSC	LGSC	EC	CCC	MC
Proposed tissue of origin	Fallopian tube fimbria or Müllerian epithelium	Fallopian tube fimbria or Müllerian epithelium	Endometriosis or atypical endometriosis	Endometriosis or atypical endometriosis	Unknown
Proposed cell of origin	Fallopian tube secretory epithelial cell or epithelial cell of Müllerian origin – ovarian CIC via endosalpingiosis or other unknown mechanism	Fallopian tube secretory epithelial cell or epithelial cell of Müllerian origin – ovarian CIC via endosalpingiosis or other unknown mechanism	Endometrial epithelial call	Endometrial epithelial call	Unknown
Precursor lesion	STIC or neoplasm of CIC	Borderline tumour	Atypical endometriosis	Atypical endometriosis	Borderline tumour
Proposed carcinogenesis	Unknown	Step-wise	Unknown	Unknown	Step-wise

Information was adapted and modified from references [37,39]. CCC: Clear Cell Carcinoma; CIC: Cortical Inclusion Cyst; EC: Endometrioid Carcinoma; HGSC: High-Grade Serous Carcinoma; LGSC: Low-Grade Serous Carcinoma; MC: Mucinous Carcinoma; and STIC: Serous Tubal Intraepithelial Carcinoma.

Table 1.3.2. The common somatic events in the main epithelial ovarian cancer subtypes.

HGSC	LGSC	EC	CCC	MC
Variants in: - *TP53* (>95%) - DNA repair genes (50%, including germline variants)	**Variants in:** - *KRAS* (10-20%) - *BRAF* (5-30%) - *USP9X* (15%)	**Variants in:** - *CTNNB1* (30-50%) - *ARID1A* (30%) - *PIK3C* (15-40%) - *KRAS* (10-30%) - *TP53* (5-25%) - *POLE* (5-10%)	**Variants in:** - *ARID1A* (40-60%) - *PIK3C* (30-60%) - *TP53* (10-15%) - *KRAS* (5-15%) - *CDKN2A* (5-10%) - *PTEN* (5%)	**Variants in:** - *KRAS* (80%) - *CDKN2A* (50%) - *TP53* (35%)
High genomic instability: - *NF1* loss (15%) - *PTEN* loss (5%) - *RB1* loss (15%) - *CCNE1* amplification (20%)	**Low genomic instability:** - *USP9X* loss (10%)	MMR deficiency and/or high microsatellite instability (5-15%)	MMR deficiency and/or high microsatellite instability (5-15%)	**Variable genomic instability:** - *CDKN2A* loss (75%) - *KRAS* and *TP53* loss (60%) - *ERBB2* amplification (25%)
Others: - Methylation of *BRCA1* (5-15%) or *RAD51C* (1-3%)	-	**Others:** - Methylation of any of the MMR genes (5-50%)	**Others:** - Methylation of any of the MMR genes (5-10%)	-

Percentages are in approximations and information was summarized from references [7,20–31,40–42]. CCC: Clear Cell Carcinoma; EC: Endometrioid Carcinoma; HGSC: High-Grade Serous Carcinoma; LGSC: Low-Grade Serous Carcinoma; MC: Mucinous Carcinoma; MMR: Mismatch Repair; and (-) No information or not applicable.

There has been a tremendous amount of effort made to investigate the origin and pathogenesis of HGSC [24,37,39,43]. The incessant ovulation hypothesis was proposed in the mid-1970s as the HGSC subtype arises de novo from the ovarian surface epithelium due to repeated rupture and repair of these cells that may lead to an increased rate of proliferation and metaplasia and accumulation of genetic events [37,44]. Two decades later, it was hypothesized that HGSC arises from cortical inclusion cysts (CICs) which are proposed to be derived from the ovarian surface epithelium during ovulation via endosalpingiosis [37,39,43,45–47]. This hypothesis was further supported by histopathological studies [48,49] to be generally accepted until the early 2000s [37,39,43]. A paradigm shift in the possible cell of origin of HGSC took place with the increase in prophylactic salpingo-oophorectomy surgeries (presented in **sub-section 1.5.1.2.**) for women harbouring germline pathogenic variants (PVs) in *BRCA1* or *BRCA2*, known OC predisposing genes (OCPGs) as presented in depth in **sub-section 1.5.2.2.** [39,43]. Striking observations were reported regarding the microscopic presence of occult tubal, non-invasive and/or invasive carcinomas in the fimbria of the fallopian tubes that were found to share the same histopathological features as HGSC cells such as high atypia, high mitoses and lack of polarity [50,51]. A follow-up report revealed that these abnormal cells of the fimbria have a similar immunohistochemical profile as HGSC cells, which resulted in proposing these cells as precursor lesions of HGSC [18,37,52]. Such cells may disseminate, become implanted and grow on the epithelial ovarian surface to ultimately result in a primary HGSC [52]. These cells were then referred to as serous tubal intraepithelial carcinomas (STICs) [37,39,43]. A series of reports have since been published to support this hypothesis [53–60]. Genomic studies supported the shared genomic characteristics between STIC and HGSC cells such as *TP53* somatic variants [40,41]**.** It has been shown that there is a clonal similarity between HGSC cells and the normal epithelial cells of the fallopian tubes, more so than the cells of the ovarian surface [41], which is further supported by in vivo studies [61–63]. All these studies together support the idea that somatic PVs in *TP53* are most likely to occur first and subsequent formation of STIC cells by an unknown mechanism, which ultimately leading to the onset of HGSC [24,64]. In spite of the promise of a prevention strategy by identifying STIC cells for women at high risk for HGSC, there is a wide range of 20-60%

of women diagnosed with HGSC with the evidence of STICs [7,37,39,65]. This suggests the need for an alternative hypothesis for the origin and pathogenesis of HGSC [37]. A dualistic model for HGSC pathogenesis has then been proposed recently to incorporate all HGSC and to correlate their clinico-pathogical and molecular profiles with their potential precursor lesions [7,37,39]. One aspect of the model is that HGSC with the presence of STICs may arise through early somatic (and/or germline) events of DNA repair pathways deficiency [37,66–68] as one of the hallmarks of cancer [69,70]. Whereas, HGSC with the absence of STICs may arise from CICs through alternate pathways during the early stages of carcinogenesis [37]. The relative proportion of HGSC cases with STIC or CICs cells is still unknown [71], yet there are reports of a small proportion of HGSC that have both CIC and STIC cells [37,72,73].

For EC and CCC, endometrial tissue has long been thought to be the tissue of their origins [37]. Endometriosis is defined as endometrial tissue growing outside the uterine cavity and it commonly occurs on the ovaries as blood-filled endometriotic cysts or on other pelvic or abdominal areas [26,37,74,75]. Approximately 10% of women are diagnosed with endometriosis with the majority diagnosed in their 20s [26,74,75]. The underlying molecular mechanism of carcinogenesis stemming from endometriosis remains poorly understood [37]. For LGSC, the current hypothesis of its origin is that it may arise from a larger tubal-type of CIC [38] in a slow, step-wise fashion from borderline tumours [39,76], while the origin is less known for MC [29,77].

1.4. Treatment of ovarian cancer

Cytoreductive surgery was the only treatment option for women with OC until the late 1970s when platinum-based chemotherapy was approved, and two decades later taxane-based chemotherapy was introduced [7,78,79]. Several clinical trials have been conducted to assess the efficacy and safety of these treatments, alone or in combination, to achieve optimal clinical outcomes [7]. Since then, chemotherapy along with cytoreductive surgery has been the standard of care for OC [7,78,79]. Generally, cytoreductive surgery is performed as the first line of treatment, with the goal of removing as much of the tumour as possible, followed by the administration of six cycles of chemotherapy: a platinum-based (cisplatin or carboplatin) and/or a taxane-based

chemotherapy (paclitaxel), with the goal to eradicate any microscopic disease remaining after surgery [7,78,80,81]. With the aim of improving the clinical outcome, the concept of neoadjuvant treatment was introduced; this regime involves undergoing first three cycles of chemotherapy to reduce the size or the extent of the tumours prior to cytoreductive surgery to remove any macroscopic tumours left, followed by an additional three cycles of chemotherapy are administered [7,78]. A series of randomized control trials were conducted to assess the clinical outcome of adjuvant versus neoadjuvant treatment with different OC groups, and revealed that adding the neoadjuvant treatment is not inferior to the adjuvant treatment, but there were lower morbidity and mortality rates in the neoadjuvant treatment group [7,78,82,83]. There is no single protocol suitable for treating all women with OC, where generally the adjuvant regime is preferred for newly diagnosed women with any OC subtype and the neoadjuvant regime can be the first choice for a subset of cases such as older women and/or those with multiple co-morbidities [7,78,84]. Over 80% of women with OC that initially respond to treatment will eventually relapse within a five-year window [7,25,38,78]. Approximate rates of response and recurrence per OC subtype are presented in **Table 1.4.1**. The grim reality is that at least 50% of recurrent OC cases will become resistant to chemotherapy [7,25,38,78]. Chemoresistance is, indeed, the most difficult issue in OC management as no alternatives can be offered [78]. This has triggered extensive research to understand the underlying molecular mechanisms of chemoresistance [85–88].

The limitations of the current chemotherapy drove research towards alternate treatments [7]. Targeted therapies were developed, of which a few that have been recently approved for OC [7,78,89,90]; these are summarized in **Table 1.4.2.** The first approved targeted therapy for OC is Bevacizumab as a monoclonal antibody that acts against angiogenesis to eventually result in apoptosis of cancerous cells [7,78,89,90]. Clinical outcomes have been assessed for bevacizumab in combination with the standard-of-care chemotherapy, and favourable clinical outcomes were observed for recurrent platinum-resistant OC cases who received bevacizumab with the chemotherapy versus chemotherapy alone [90]. Bevacizumab has become the first-line therapy for recurrent platinum-resistant OC cases [7,78,89,90]. The breakthrough targeted therapy for OC is poly (ADP-ribose) polymerase (PARP) inhibitor [78,90,91].

Table 1.4.1. The characteristics of prognosis and treatment response for the main epithelial ovarian cancer subtypes.

	HGSC	LGSC	EC	CCC	MC
Overall response rate to platinum and/or taxane-based chemotherapy	>70%	<10%	>60%	<15%	20-60%
Recurrence rate within five years	>50%	<30%	60-40%	20-60%	6-20%

Percentages are approximations and information was summarized from references [26,28,36,92]. CCC: Clear Cell Carcinoma; EC: Endometrioid Carcinoma; HGSC: High-Grade Serous Carcinoma; LGSC: Low-Grade Serous Carcinoma; and MC: Mucinous Carcinoma.

Table 1.4.2. Approved targeted treatment for epithelial ovarian cancer subtypes.

Targeted inhibitor	First approval	Approved in Canada	Molecular pathway	General structure	Overall underlying mechanism	Clinical name(s)	Targeted OC cases
VEGF inhibitors	2014	2017	Angiogenesis	Monoclonal antibody	Binding with VEGF	Bevacizumab	Poor prognosis for advanced stages, recurrent platinum-sensitive and resistance
PARP inhibitors	2014	2020	HR DNA repair	Chemical compound	Synthetic lethality with HR deficiency	Olaparib, Rucaparib, Niraparib, Talazoparib, Veliparib, Pamiparib, Fluzoparib	Recurrent platinum-sensitive

Information was summarized from references [89,90] and (fda.gov; and canada.ca/en/health-canada.html). HR: Homologous Recombination; PARP: Poly (ADP-Ribose) Polymerase; and VEGF: Vascular Endothelial Growth Factor.

This chemical compound acts through synthetic lethality; PARP inhibitors inhibit the single-stranded DNA break repair machinery, resulting in the accumulation of double-stranded DNA breaks which are mainly repaired by the homologous recombination (HR) pathway via BRCA1, BRCA2 and other proteins [91,93]. Synthetic lethality occurs in the cancerous cells that are deficient for *BRCA1*, *BRCA2* or, theoretically, any of the protein-encoding genes that is involved in HR [91,93]. Clinical trials have been conducted to assess olaparib as the first approved PARP inhibitor alone and in combination with the standard-of-care chemotherapy [90,91]. Strikingly favourable clinical outcomes were observed for recurrent OC cases who tested positive for germline PVs in *BRCA1* or *BRCA2* versus those in the placebo arm [90,91]. Comparable outcomes were also reported for OC cases positive for somatic PVs in *BRCA1* or *BRCA2* as well as for cases positive for germline or somatic PVs in other genes involved in the HR pathway [78,90], and recently for cases positive for *BRCA1* promoter hypermethylation [94]. Other PARP inhibitors have been produced for better efficacy and less toxicity and are currently in clinical trials [90,91].

1.5. Risk factors associated with ovarian cancer

Today, there is still no effective screening for OC, which explains why the majority of epithelial OC cases of up to 90% of women with HGSC subtype specifically present clinically at advanced stage [7,95,96]. Current clinical examination for OC is performed by transvaginal ultrasonography and by measuring Cancer Antigen 125 (CA125) serum levels, which are elevated in non-cancerous disorders such as ovarian cysts and endometriosis [7,96]. On the other hand, there are few epidemiological and heritable genetic risk factors that are associated with OC risk [4,5,7,10]. The causality of these factors in increasing or modifying risk for developing OC remains unknown, yet several hypotheses have been proposed to elucidate the underlying mechanisms in ovarian carcinogenesis [4,97,98].

In the following sub-sections, I presented briefly the associated epidemiological factors in **sub-section 1.5.1.** and the heritable genetic factors in **sub-section 1.5.2.** Only a few rare heritable genetic risk factors have been documented to have a large effect size, and thus, have been implemented in clinical management [99,100].

A periodic assessment of potential OC risk factors is performed by a multidisciplinary team, and only those with sufficient evidence, so-called evidence-based medicine, are implemented for clinical management [99,101] (cebm.ox.ac.uk).

1.5.1. Epidemiological risk factors

Here, I summarized the epidemiological risk factors as those associated with an increased risk in **sub-section 1.5.1.1.** and those associated with a reduced risk for developing OC in **sub-section 1.5.1.2.** Other factors with controversial associations are briefly summarized in **sub-section 1.5.1.3**.

1.5.1.1. Risk-increasing epidemiological factors

Genetic factors: Family history of ovarian cancer and other cancers

Having a family history of OC and/or other cancers is a well-established epidemiological risk factor for developing OC [4,5,7,102,103]. The incidence of OC, however, was not increased based on studying 3,072 first-degree relatives of 559 unselected OC cases, where the standardised incidence ratio (SIR) in females was 1.0 (95% CI: 0.8 to 1.1) [104]. Female relatives had a significantly increased risk for OC (SIR: 2.8; 95% CI: 1.8 to 4.2), and the relative risk (RR) for developing OC was found to be age-dependent [104]. It was shown that there is a statistical difference in the RRs for developing OC for women having a first-degree relative with an OC diagnosed before the age of 50 years (RR: 4.72; 95% CI: 3.21 to 6.95) compared to those who were diagnosed at an older age (RR: 2.53; 95% CI: 1.91 to 3.35) [105]; this trend was consistent with previous reports [105–107]. It was also reported that the RR of developing OC differs depending on whether a woman has a relative diagnosed with serous (RR: 3.64; 95% CI: 2.27 to 4.87) versus one diagnosed with non-serous (RR: 2.25; 95% CI: 1.56 to 3.26) [105], which is consistent with a previous study [108].

Familial risk for developing OC varies among women according to the degree of relationship and the number of relatives with OC [4,5,7,109–111]. A recent population-based study reported that the RR of developing OC for a woman with a first-degree relative with OC is approximately three-fold (RR: 2.96; 95% CI: 2.35 to 3.72) compared to the general population, which is consistent with other studies [104,106,107,112].

Interestingly, the authors also reported that the RR is comparable to those with first-degree relatives with OC testing positive versus those testing negative for PVs in *BRCA1* or *BRCA2* (RR: 2.24; 95% CI 1.71 to 2.94) [104,105,113,114]. There is no statistical difference in the estimated RRs among mother-proband, daughter-proband or sister-proband comparisons, which has been controversial among previous studies [104–107,112]. A family-based study reported that the RR decreases dramatically for a woman who has one second-degree relative with OC (RR: 2.78; 95% CI: 0.31 to 10.0) compared to a woman having one first-degree relative with OC (RR: 10.09; 95% CI: 5.03 to18.1) [115]. Moreover, a meta-analysis study estimated that the pooled RRs of developing OC for a woman having at least two or more OC cases in first- and/or second-degree relatives is 11.7 (95% CI: 5.3-25.9) [106].

While a family history of OC is a risk factor for developing OC, having relatives with breast cancer (BC) or other cancers is also an indicator for an increased risk for developing OC [7,104,106,107,109–112]. The incidence of OC among sisters with a family history of BC was found to be significantly higher (SIR: 9.2; 95% CI: 3.7 to 19.0) than among sisters without a family history of BC (SIR: 2.9; 95% CI: 1.6 to 4.8) [104]. The RR of developing OC for a woman having a first-degree relative with BC was estimated to be 1.7 (95% CI: 0.8 to 3.0), and the risk increases up to 2.3 (95% CI: 0.9 to 3.9) with an increasing number of first-degree relatives with BC [107,110]. It was also reported that OC cases are clustered significantly with other cancers such as pancreatic, prostate and colorectal cancers [110], yet the risk of a woman developing OC with close relatives with these cancers is unknown. Currently, women with a family history of OC and/or BC are not recommended for risk-reducing bilateral salpingo-oophorectomy (RRSO) as they do not meet the threshold of actionability of absolute risk more than 4% [99] (presented in following **sub-section 1.5.1.2.**). However, the magnitude of such risk can be influenced by several factors such as harbouring a heritable genetic risk factor (presented in **sub-section 1.5.2.2.**), and subsequently the absolute risk is adjusted for considering the procedure [99,101,105].

Demographic factors: Age and ethnicity

Age is a well-documented risk factor for developing epithelial OC where the disease is positively correlated with advanced age [1,3,5,9]. However, it has been reported that age is not an independent prognostic factor [5,116]. The underlying mechanism of how advanced age predisposes women to developing OC is unknown [5]. Ethnicity is another risk factor for developing OC although its causality is unclear [117–119].

Gynecologic or reproductive related risk factors

Endometriosis has been consistently associated with the increased risk for developing the specific epithelial OC subtypes, EC and CCC [74,75]. A meta-analysis of 49 population-based studies confirmed this association with CCC (RR: 3.44; 95% CI: 2.82 to 4.42) and EC (RR: 2.33, 95% CI: 1.82 to 2.98), but not with the other OC subtypes [120]. Despite the strong link between endometriosis and OC, the link between other gynecologic-related risk factors such as pelvic inflammatory, ovarian cysts and ovarian hyperstimulation treatment for infertility and OC have been controversial [4,5,7,119].

1.5.1.2. Risk-reducing epidemiological factors

Gynecologic factors: Salpingo-oophorectomy and tubal ligation

The association of unilateral or bilateral salpingo-oophorectomy (BSO) with the lifetime reduction in risk of developing OC is well-documented [4,5,7,121,122]; over 50 clinical trials have been conducted to assess RRSO in the context of OC risk (canadiancancertrials.ca). A meta-analysis of 77 studies reported that BSO reduces the risk of developing OC by approximately 51% (95% CI: 0.35 to 0.75) [123]. A recent population-based study revealed that BSO was associated with an absolute risk reduction of 0.38% (95% CI: 0.32 to 0.45) for OC and 0.18% (95% CI, 0.11 to 0.25) for OC deaths, based on over 20 years of follow-up data [124]. A comparable absolute risk reduction of OC was reported for women harbouring *BRCA1* PVs (Hazard Ratio [HR]: 0.30; 95% CI: 0.24 to 0.38) or *BRCA2* PVs (HR: 0.33; 95% CI: 0.22 to 0.5) [125]. The impact of RRSO extended beyond OC, with evidence that *BRCA1* or *BRCA2* PV carriers who undergo the procedure also had a significant reduction in the development of BC (HR: 0.58; 95% CI: 0.37 to 0.78) [126], which is consistent with a previous prospective

study [127], yet the mechanism underlying this effect remains unclear [128]. The RRSO procedure itself was shown to be safe [129], and can be considered if a woman has a greater than 4% absolute risk of developing OC, the current clinical threshold [99]. Based on the current National Comprehensive Cancer Network® (NCCN) Clinical Practice Guidelines in Oncology - Guidelines of the Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic – Version 2.2022 (nccn.org/guidelines/category_2), RRSO is routinely recommended to women harbouring PVs in *BRCA1* or *BRCA2* to reduce the risk of developing OC [99,101,128]. In cases negative for *BRCA1* and *BRCA2* PVs, careful consideration is required to ensure that benefits of the procedure outweigh the associated risks such as premature menopause and its associated symptoms (presented in **sub-section 1.5.2.2.**).

Tubal ligation is also associated with OC risk reduction [4,5,7]. A population-based study of over one million women showed that tubal ligation is associated with OC risk reduction (RR: 0.80, 95% CI: 0.76 to 0.85) [130]. A plausible hypothesis to elucidate the underlying molecular mechanism of such protective effect is that tubal ligation may mechanically block cancer precursor cells and/or chemicals from reaching the surface of the ovary, fallopian tubes or peritoneal cavity [37,75,131]. However, association of tubal ligation with OC risk reduction among women harbouring *BRCA1* or *BRCA2* PVs has been controversial [4,5,7]. A population-based study reported that tubal ligation is associated with the reduced risk of OC among women harbouring *BRCA1* PVs (HR: 0.42; 95% CI: 0.22 to 0.80) [132], which is consistent with a meta-analysis study [133].

Reproductive factors: Oral contraceptives (OCPs)

OCPs have become the primary protective risk factor against developing OC [5,7,10,134–136]. A meta-analysis study showed that there is a significant reduction in OC risk among women who had used OCPs versus those who had never used them (OR: 0.73; 95% CI: 0.66 to 0.81), and with more than 50% risk reduction among 10 year-users or more [11]. A similar effect was also observed among OCPs users harbouring PVs in *BRCA1* or *BRCA2* (OR: 0.58; 95% CI:, 0.46 to 0.73) [132,137]. The observed protective effect not only increases with the increased duration of use [138], but also continues to increase up to 35 years after being discontinued [139]. This may

explain the decrease in the OC incidence rates in North America and some of the European countries where OCPs are widely used [1,10]. The underlying molecular mechanism of the protective effect of OCPs is unknown, one proposed hypothesis is that OCPs, which is containing a lower standard dose of synthetic estrogen and progestin, may exert a protective effect by acting directly on the fallopian tube secretory cells, which express estrogen and progesterone receptors [37]. Other hypothesis is that OCPs are acting indirectly by preventing the monthly exposure of the epithelial cells at the surface of the ovary and the fimbria of the fallopian tubes to the estrogen-rich follicular fluids and inflammatory microenvironment that follows ovulation, also known as the incessant ovulation hypothesis [38,44]. In contrary, OCPs are associated with an increased risk of developing BC [139] as well as other adverse cardiovascular-related events [136]. This poses a dilemma for recommending the use of OCPs to reduce the risk of developing OC [136].

Other reproductive factors: Menarche, parity and breastfeeding

Several reproductive related factors have been reported to be associated with reduced risk of developing OC [4,5,7,10,119] such as older age of menarche [140], older age at last childbirth [141], parity of one versus three or more and duration of breastfeeding [142]. Even though the underlying mechanism of the protective effect of these factors is unknown, this may be explained by the incessant ovulation hypothesis [5,44]. The continuation of ovulation by any of the factors mentioned above may contribute to the reduced risk of developing OC by preventing the repeated mechanical damage and exposure of the epithelial cells of the ovary and the fimbria of the fallopian tubes to the estrogen-rich follicular fluids and inflammatory microenvironment [38,44].

1.5.1.3. Other controversial epidemiological factors

The association of hormone replacement therapy with OC risk has been controversial [4,5,7] with regards to the type and/or the duration of hormonal replacement used [143–149]. The association of lifestyle and environmental factors such as nutrition, obesity, sedentariness, smoking, asbestos and talcum powder exposure, aspirin and nonsteroidal anti-inflammatory drug use with OC risk have been controversial [4,5,7].

1.5.2. Heritable genetic risk factors

In addition to the epidemiological risk factors, heritable genetic risk factors have also been shown to be associated with OC risk. OC is shown to have a strong heritable component which is estimated to be at approximately 39% (95% CI: 23 to 55) based on a twin study [102]. Hereditary OC cases account for approximately 20-25% of all epithelial OC cases, of which less than 5% are familial, a clustering of close relatives with OC [7,109,110,150,151]. Heritable genetic risk factors are, hence, inherited from either one or both parents, depending on the mode of inheritance of the cancer [152,153]. Harbouring such heritable genetic risk variant predisposes the individual to develop cancer during their lifetime [152,153]. These risk variants can be classified as common or rare based on their frequencies in the general, non-cancer population; the associated RR of harbouring such genetic risk variant is generally classified into: (1) high risk (RR >5); (2) moderate risk (RR ≥2 and ≤ 5) or (3) low risk (RR <2) [154–156], though these thresholds are arbitrarily defined [156,157].

In the following sub-sections, I presented the common low-risk genetic factors known to play a role in OC in **sub-section 1.5.2.1.** and rare moderate- to high-risk genetic factors in **sub-section 1.5.2.2.**

1.5.2.1. Common low-risk genetic factors

Common genetic risk variants are generally defined based on their frequencies in the general population by having a minor allele frequency (MAF) of more than 5% [97]. Because of the relatively high prevalence of these common variants in the population and the relatively low frequency of OC, the effect size of these variants is estimated to be small [158,159]. The contribution of these common low-risk variants collectively account for approximately 4-6% of OC cases [97]. These common risk variants are usually identified by genome-wide association studies (GWAS) [158,159], which also determine the additive effect of multiple common risk variants under a polygenic model, so-called a polygenic risk score (PRS) [160]. Few population-based studies have been conducted to assess the association of PRS with OC risk as summarized in **Table 1.5.1.** As these GWAS studies are usually performed on thousands of cases and controls, a national and/or international consortium is required to collect a large number of

participants [158,159]. The Ovarian Cancer Association Consortium (OCAC) (ocac.ccge.medschl.cam.ac.uk), for instance, is a multidisciplinary forum to investigate the genetic and epidemiological risk factors of OC among over 20,000 OC cases of different histopathological subtypes and controls, mainly from the European population [158,159]. Almost all OC risk loci known today have been identified by the OCAC [97]. To date, there are more than 30 loci associated with increased OC risk in the European population [4,97,161]. Only one distinct OC risk locus was reported by a GWAS that was conducted on the Han Chinese population [97,162]. Some of these loci are associated with the overall OC risk, while others are associated with specific OC subtypes: ten are associated with HGSC; nine with MC, LGSC or serous borderline; one with EC; and none with CCC [4,97,161]. The highest reported association with OC risk was for a locus in chromosome region 8q21.11 (OR: 2.19; 95% CI: 1.65 to 2.90), which is associated with LGSC and serous borderline, and the lowest for a locus in region 8q24.21 (OR: 1.08; 95% CI: 1.05 to 1.11), which is associated with HGSC [4,97,163]. Two loci were reported to be associated with the overall reduced OC risk in region: 17q12 (OR: 0.79; 95% CI: 0.73–0.86) in the European population, and 10p11.21 (OR: 0.81; 95% CI: 0.70–0.95) in the Han Chinese population [4,97,162,163]. Some loci have been reported to be associated with OC and other cancers such as a locus in region15q26.1 is associated with OC and BC risk, one in region 11q12.3 is associated with OC, BC and prostate cancer, and another on in region 8p24 was reported to be associated with more than ten different cancers, including OC, which is referred to as a pleiotropic locus [97,164]. All these OC risk loci are intronic or intergenic, yet their biological impact remains unknown [97,161,165]. These common OC risk loci are not clinically implemented for OC risk assessment and clinical management based on the current clinical guidelines due to their small effect size [97,99,159,166,167].

1.5.2.2. Rare moderate- to high-risk genetic factors

Under the polygenic model, the heritable genetic component of OC is far to be explained by common risk loci, whereas it is largely explained by rare risk variants under a monogenic model [168]. Though the initiation and progression of most OC cases are primarily driven by the acquisition of somatic genetic or epigenetic events, so-called

Table 1.5.1. Summary of polygenic risk score-based studies in ovarian cancer.

Year when the study was conducted	Study type	Number of OC risk loci	Number of cases (*BRCA1* and *BCA2* status)	Number of controls	Ethnicity	Estimated risk of PRS with OC	95% CI	Reference
2018	Case-control	15	750 (Regardless of *BRCA1* and *BRCA2* PVs status)	1,428	European	1.32 of OC 1.43 of SC	1.21 to 1.45 1.29 to 1.58	[166]
2020	Cohort	313	18,935 *BRCA1* 12,339 *BRCA2*	NA	European	1.32 of OC *BRCA1* PV-positive 1.44 of OC *BRCA2* PV-positive	1.25 to 1.40 1.30 to 1.60	[169]
2022	Case-control	27,240	23,564 (Regardless of *BRCA1* and *BRCA2* PVs status)	40,138	European, East Asian and African	1.38 of OC of European 1.14 of OC of East Asian 1.38 of OC of African	1.28 to 1.48 1.08 to 1.19 1.21 to 1.58	[170]

CI: Confidence Interval; NA: Not applicable; OC: Ovarian Cancer; PRS: Polygenic Risk Score; PV: Pathogenic Variant; and SC: Serous Carcinoma.

cancer drivers, the initiation of a subset of cases is a consequence of harbouring germline PVs in moderate- or high-cancer predisposing genes (CPG) in a tissue-specific context [37,165,171–176]. A CPG is defined by the occurrence of a rare germline genetic event in a gene increases an individual's risk for developing a specific type of cancer, or multiple primary cancers, under the two-hit hypothesis, also known as the Knudson hypothesis as proposed by Alfred Knudson in 1971 [153,165,176–179]. Under the two-hit hypothesis, a classical CPG behaving as a tumour suppressor is inactivated by a loss-of-function (LoF) genetic event of one allele at the germline level (first hit) and another hit to the other allele at the somatic level (second hit) which subsequently abrogates the biological function of the gene [165,173–179]. There is a diversity in the inactivation mechanisms of CPGs, of which the majority behaving as classical tumour suppressors that require both alleles to be inactivated, while haploinsufficiency and dominant-negative mechanisms also occur [165,177–179]. In some hereditary cancers, both hits occur at the germline level (autosomal recessive inheritance), whereas one hit at the germline is sufficient to increase the susceptibility of an individual for developing cancer in the majority of hereditary cancers including OC (autosomal dominant inheritance) [153]. As the majority of CPGs behave as tumour suppressors, there are less than 10% that behave as oncogenes that require a gain-of-function genetic event and subsequently activate its biological function [168,179].

In the following paragraphs, I summarized known OC risk genes chronologically and in the context of my thesis project timeline for: (1) their identification as OCPGs; (2) their biological function; (3) the contribution of PVs in these genes to OC and the type of these PVs; (4) the clinical characteristics of women harbouring PVs in OCPGs; and finally (5) the associated risk of harbouring a PV in OCPGs for developing OC or other cancers based on the NCCN Clinical Practice Guidelines in Oncology - guidelines of the Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic – Version 2.2022 (nccn.org/guidelines/category_2). These genes, and other candidates OC risk genes, are subjected for annual assessment for their associated cancer risks for clinical management [99].

Clinically actionable, known OCPGs: BRCA1 and BRCA2

BRCA1 [180] and *BRCA2* [181] were identified as BC and OC risk genes in 1994 and 1995, respectively, by classical genome-wide linkage analyses. The paradigm of genome-wide linkage analyses and positional cloning approach for the identification of *BRCA1* and *BRCA2* was recently reviewed by Dr. Patricia Tonin's group [182]. A summary of the study design for the identification and the genomic characteristics of both genes are presented in **Table 1.5.2.** and **Table 1.5.3.**, respectively.

The effort to understand the biological function of *BRCA1* and *BRCA2* started with their identification as CPGs [183]. BRCA1 and BRCA2 encoded proteins are involved in the repair of double-stranded DNA breaks via the HR pathway; the BRCA1-BARD1 heterodimer interacts with PALB2-BRCA2 to recruit RAD51 and its paralogues for a BRCA2-mediated filament formation [151,184]. Other biological functions of BRCA1 and BRCA2 have been documented such as their role in cell cycle checkpoints and transcript regulations [183,185–188].

BRCA1 and *BRCA2* are the major OCPGs, meaning PVs in either of these genes account for the majority of hereditary OC or BC families and cases [100]. The contribution of PVs in these genes, however, varies based on the family history of cancer and the population studied [100,182]. Generally, families with multiple women diagnosed with OC and/or BC, which are referred to as hereditary breast and ovarian cancer (HBOC), are more likely to be positive for PVs in either *BRCA1* or *BRCA2* than those cases not selected for family history of cancer, who are referred to as sporadic cases in this thesis [94,109]. The proportion of HBOC families positive for PVs in *BRCA1* or *BRCA2* combined ranges from 35-85% and 5-15% of sporadic OC cases [100,111,189,190] (**Table 1.5.4.**). Over 2,700 reports have been submitted on germline variants in *BRCA1* and/or *BRCA2* (PubMed access on 26 July, 2022), with a dramatic increase of submissions in 2015 when gene-panel testing was implemented [191]. Over 3,000 PVs have been reported in *BRCA1* or *BRCA2* combined, of which more than 75% are LoF variants such as frameshift, nonsense or canonical alternative splicing in 29,700 OC and BC families from 50 countries [192]. Of these families, approximately 12% are germline copy number variations (CNV) with the majority observed in *BRCA1* [192]. The contribution of *BRCA1* and *BRCA2* missense PVs remains small, at approximately 5%,

yet the effort in investigating the biological impact of CNVs and missense variants in both genes is growing with the development of increasingly sophisticated multiplex in cellulo assays [193–203]. The constitutional *BRCA1* Methylation has been recently reported to 9.4% of 461 sporadic HGSC cases [42]. Deficiency in the function of *BRCA1* or *BRCA2* eventually leads to HR-deficient tumours which is typically characterized by genomic instability [204,205]. The same phenotype has been reported in the context of germline or somatic PVs or CNVs in either of these genes or promoter methylation of *BRCA1* [94]. The average age at diagnosis of OC for women harbouring a PV in *BRCA1* is 51.3 years [range: 33–84], which is significantly younger than those harbouring PVs in *BRCA2* (61.4 years [range: 44–80]) [206] as shown in **Table 1.5.4.** The average age at diagnosis of OC for women harbouring missense PVs is comparable to those harbouring LoF variants, yet the associated OC risk of PVs, regardless of the variant type, is variable [207,208]. Part of this variability is the location of PVs in the coding regions of *BRCA1* and *BRCA2*; it has been reported that there is one OC cluster region of PVs in *BRCA1* and three in *BRCA2* that are associated with a higher risk for developing OC than BC [208]. PVs in *BRCA1* and *BRCA2* have recently been implicated in other cancers as shown **Table 1.5.5.**

A tremendous amount of effort has been conducted during the past decades to estimate the associated risks of *BRCA1* and *BRCA2* for developing OC, BC and other cancers [183,209,210]. The associated absolute risk for women harbouring PVs in *BRCA1* or *BRCA2* for developing OC are presented in **Table 1.5.6.** based on the current NCCN clinical practice guidelines in oncology (nccn.org/guidelines/category_2) [99]. The cumulative risk for developing OC by age 80 was estimated to be 44% (95%CI: 36 to 53) for women harbouring PVs in *BRCA1* and 17% (95% CI: 11 to 25) for those harbouring PVs in *BRCA2* [211]. RRSO is routinely recommended (actionability) for women between age 35 and 40 years harbouring PVs in *BRCA1*, and delaying the procedure until age 40-45 years for those harbouring *BRCA2* PVs [99,101,212,213]. Clinical management for *BRCA1* or *BRCA2* PVs carriers with other cancers are recommended [99,111,213,214].

Other known OCPGs: Mismatch Repair (MMR) genes

Other genes that have been implicated in OC risk are *MSH2* [215]*, MLH1* [216]*, MSH6* [217] and *PMS2* [218] that were identified in the 1990s. These genes were first identified as CPGs to hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch Syndrome (LS), by the classical genome-wide linkage analysis or by candidate gene approach [109,179,219]. The approaches that facilitated their identification as CPGs are summarized in **Table 1.5.2.**, and the genomic characteristics of these CPGs are presented in **Table 1.5.3.**

These MMR genes are involved in the repair of single-stranded DNA breaks via the MMR pathway [151,219]. Their encoded proteins promote genome stability by correcting mismatched DNA base pairs during replication [151,219]. These mismatched base pairs are recognized by a MSH2-MSH6 heterodimer which recruits a MLH1-PMS2 heterodimer to the faulty site for subsequent excision via EXO1, an exonuclease that permits single-strand resynthesis and ligation by DNA polymerase [151,219].

After the discovery of MMR genes in the context of HNPCC/LS families, it was observed that these genes were also associated with specific extra-colonic cancers, including OC [220] as shown in **Table 1.5.5.** Subsequent reports confirmed the association of OC with LS, and further showed that the clinical and histopathological characteristics of such cases are distinct from those harbouring PVs in *BRCA1* or *BRCA2* [109,219,221]. Women harbouring PVs in any of the MMR genes are at increased risk for developing OC, with a tendency to develop the EC or CCC subtypes, and are more likely to have OC at an early age (40s to early 50s) [100,222,223]. PVs in MMR genes account for 5-10% and less than 1% of LS and HBOC families, respectively, and less than 0.5% of OC cases not selected for family history of cancer [100,224] as shown in **Table 1.5.4.** The contribution of PVs in *MSH2* or *MSH6* to OC risk is three times higher than those in *MLH1* or *PMS2* [224]. The majority of the PVs in any of the MMR genes are LoF variants (nonsense) and less than 30% are missense PVs [225]. These PVs are distributed randomly in these genes [225], and no genotype-phenotype association has been reported for any of the MMR genes and an associated hereditary cancer syndrome [226]. Germline CNVs in any of the MMR genes, including those in *EPCAM* alone or in part of the downstream promoter region of *MSH2* [227,228],

are rarely associated with OC [229]. Deficiency in the function of any of the MMR proteins eventually leads to microsatellite instability in the tumour tissue, that is, changes in the number of the repeated DNA sequence [151,219,230].

As the contribution of PVs in any of the MMR genes in OC families and cases is small relative to those harbouring PVs in *BRCA1* and *BRCA2,* estimating the cumulative risks for developing OC among carriers of MMR variants have been challenging [99]. The current estimated absolute risk for developing OC of women harbouring a PV in *MLH1* or *MSH2* is 10%, which is higher than that for carriers of PVs in *MSH6* (3-10%) and *PMS2* (3%) (**Table 1.5.6.**) and higher than that of the overall risk for developing OC in the general population (1.2%) [3]. The current evidence for the associated risk of harbouring PVs in any of the MMR genes for developing OC remains insufficient for recommending RRSO, mainly due to the variation in the current estimated OC risks for each MMR gene [99]. Rather, the discussion of offering RRSO and hysterectomy is conducted with these women in a gene-specific manner, keeping in mind that carriers of PVs in *MLH1*, *MSH2* or *MSH6*, for instance, are also at increased risk for developing endometrial cancer, unlike for carriers of PVs in *PMS2* [99].

Other relatively new OCPGs: RAD51C, RAD51D and BRIP1

RAD51C, RAD51D and BRIP1 were first reported as OCPGs five to six years before I started my Ph.D. in 2016, hence, I defined them here as relatively new OCPGs to the previously mentioned OC risk genes. Both *RAD51C* [231] and *RAD51D* [232] were reported as OCPGs in 2010 and 2011, respectively, using a candidate gene approach focused on genes involved in the HR pathway. *BRIP1*, on the other hand, was originally reported as a BC risk gene in 2006 also using a candidate gene approach [233], and a few years later in 2011, it was reported to be an OCPG using a whole-genome association case-control study [234]. The applied approaches that facilitated the identification of *RAD51C, RAD51D* and *BRIP1* as CPGs are summarized in **Table 1.5.2.**, and the genomic characteristics of these CPGs are presented in **Table 1.5.3.**

RAD51C, RAD51D and BRIP1 encoded proteins are involved in HR [151]. RAD51C and RAD51D are involved in the repair of double-stranded DNA breaks by mediating RAD51 filament formation for proper strand inversion and repair in a BRCA2-

dependent manner; this occurs by forming two complexes of heterodimers: RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) and RAD51C-XRCC3 (CX3) [151,184,186,235–237]. BRIP1, on the other hand, is a BRCA1 binding partner and depends on this interaction for its recruitment and stabilization [151].

Since the initial reports of the identification of *RAD51C, RAD51D* and *BRIP1* as OCPGs, a number of studies on germline variants in these genes in the context of hereditary OC have been reported [100,238]; over 800 reports have been submitted on germline variants in either of these genes (PubMed access on 26 July, 2022), with a dramatic increase of submissions in 2015 when gene-panel testing was implemented [191]. The average age at diagnosis of OC among women harbouring PVs in *RAD51D* is in their late-50s and is comparable to those harbouring PVs in *BRCA2*, whereas the age of diagnosis of carriers of PVs in *RAD51C* or *BRIP1* is in their early-to-mid 60s and is comparable to those of the general population [239] (**Table 1.5.4.**). The majority of the PVs in *RAD51C, RAD51D* and *BRIP1* are LoF variants (frameshift, nonsense and canonical alternative splicing) and less than 2% are missense PVs [240]. as shown in **Table 1.5.4.**, the contribution of PVs in these genes among families with at least one OC case is less than 5%, and this proportion increases to approximately 12% for families with at least two or more OC cases versus less than 2% of carriers of PVs with OC not selected for family history of cancer [100]. PVs are distributed across these genes [240], with a possible genotype-phenotype association reported for PVs in *BRIP1* and OC risk [241], but not for *RAD51C* or *RAD51D* [238]. Germline CNVs are rare, less than 0.5%, in any of these genes in OC cases [229]. Deficiency in the function of *RAD51C* or *RAD51D* eventually leads to HR-deficient tumours, analogous to those with deficiencies in *BRCA1* and *BRCA2* [204,205]. Interestingly, the same phenotype has been reported in the context of promoter methylation of *RAD51C* as for *BRCA1* [94]. PVs in *RAD51C, RAD51D* or *BRIP1* have recently been implicated in other cancers as shown **Table 1.5.5.**

As the contribution of PVs in *RAD51C, RAD51D* and *BRIP1* in OC families and cases is small relative to those harbouring PVs in *BRCA1* and *BRCA2,* estimating the cumulative risk for developing OC among carriers of these new genes has been challenging. Additionally, it is important to emphasize that the risks presented here are

predominantly based on LoF variants in *RAD51C, RAD51D* or *BRIP1,* and the risks for missense PVs has yet to determined [242]. The first two population-based studies to estimate the cumulative risk of developing OC for individuals harbouring *RAD51C, RAD51D* [33] or *BRIP1* [241] PVs were reported in 2015 and were found to be 1.3% (95% CI: 0.3 to 6.0) and 5.2% (95% CI: 1.1 to 22) for *RAD51C* by age of 50 and 70, respectively; 3.0% (95% CI: 0.4 to 21) and 12% (95% CI: 1.5 to 60) for *RAD51D* by age of 50 and 70, respectively; and 5.8% (95% CI: 3.6 to 9.1) by age of 80 for *BRIP1*. These findings have since been replicated in a recent large study of over 30,000 OC cases and reported the ORs for carriers of PVs: in *RAD51C* (OR: 5.59; 95% CI: 4.42 to 7.07), in *RAD51D* (OR: 6.94; 95% CI: 5.10 to 9.44) and in *BRIP1* (OR: 4.94; 95% CI: 4.07 to 6.00) [240]. In a familial context, the cumulative risk for developing OC was found to be 11% (95% CI: 6% to 21%) among *RAD51C* and 13% (95% CI:7% to 23%) among *RAD51D* PV carriers by the age of 80 [243]. The estimated cumulative risk can be as high as 32 to 36% when two first-degree relatives are diagnosed with OC [243]. This risk increased with age until around age 60 years and decreased thereafter [243]. As summarized in **Table 1.5.6.**, the absolute risk of developing OC for a carrier of a PV in *RAD51C, RAD51D* or *BRIP1* is at least 10% compared to 1.2% for the general population [99]. Based on the current guidelines for OC clinical management, RRSO is recommended for carriers of PVs in any of these three genes, though these risks are under annual assessment to update their Evidence-Based Medicine Quality Rating [99,212].

Other new OCPGs: ATM, PALB2 and others

ATM and *PALB2* were both first identified as BC risk genes using a candidate gene approach [244,245]. The applied approaches that facilitated their identification as CPGs and the genomic characteristics of both genes are presented in **Table 1.5.2.** and **Table 1.5.3.**, respectively.

ATM is a protein kinase that plays a critical role in monitoring double-stranded DNA repair via the FA and HR pathways [151,184,246]. PALB2, on the other hand, is required to recruit and stabilize BRCA2 at DNA double-stranded break sites for repair via the HR pathway [151,247]. Deficiencies in the function of *PALB2* and *ATM*

eventually lead to HR deficient tumours, similar to what is observed in the case of deficiencies in *BRCA1*, *BRCA2*, *RAD51C* and *RAD51D* [204,205,248–250].

Since their identifications as CPGs, *ATM* and *PALB2* have been consistently implicated in BC risk, resulting in their inclusion in gene-panel tests [156]. Subsequently, *ATM* and *PALB2* were implicated as plausible OCPGs as well [34,251–254]. The main limitations of these studies were the small sample size and/or the absence of appropriate controls [100]. *PALB2* was first investigated as a candidate OCPG in 2015 in a European population-based study where its coding and flanking intronic regions were sequenced for less than 4,000 OC cases and matched-controls. While no significant statistical difference in the frequency of PVs between cancer cases versus controls was observed, a marginal association was detected [241]. This suggested that a larger number of samples is required to determine whether *PALB2* is an OCPG [100]. This was indeed confirmed in a recent report using a candidate gene approach combined with targeted sequencing of 54 genes involved in DNA repair or whole-exome sequencing (WES) data of over 6,000 OC cases and controls and genotyping data from an independent set of approximately 14,000 OC cases and 28,000 controls, all of European origin [255]. In contrast, the association of harbouring a PV in *ATM* in a heterozygous state with OC was first reported in 2017 by gene-panel testing of 5,000 OC cases and controls predominantly of European origin [256], and was further confirmed by subsequent studies [257–260]. The frequency of OC cases carrying PVs in these genes is generally small in families and sporadic cases as shown in **Table 1.5.4.** As these genes are strongly associated with BC risk, it is not surprising to observe a family history of individuals with BC for these OC cases [100,256,257]. The PVs are distributed across the *ATM* and *PALB2* genes [247,258], and germline CNVs are generally rare (less than 0.5%) [229]. PVs in *ATM* or *PALB2* have also been also implicated in other cancers as shown **Table 1.5.5.**

As summarized in **Table 1.5.6.**, the absolute risk of developing OC of harbouring a PV in *ATM* or *PALB2* in a heterozygous state is 5% or lower by age 70 in contrast to an absolute risk of 15-60% for developing BC in such carriers [99]. Based on the current guidelines for clinical management of OC, there is insufficient evidence to recommend

Table 1.5.2. The approach used in the identification of the clinically actionable known and other ovarian cancer predisposing genes.

OC risk gene	Year of the gene identification (Reference)	Genetic approach	Population ethnicity[1]	Number and type of families or cases	Study group selection (*BRCA1* or *BRCA2* status)	Study methodology
BRCA1	1994 [180]	Linkage analysis	Mixed	8 BC and OC cases from HBC and/or HBOC families	Multi-generation pedigrees of affected and unaffected family members (unknown *BRCA1* or *BRCA2* PVs status)	Cloning, cDNA-based PCR, and sanger sequencing
BRCA2	1995 [181]	Linkage analysis	Mixed	6 BC and OC cases from HBC and/or HBOC families	Multi-generation pedigrees of affected and unaffected family members (*BRCA1* PV-negative)	Same as above
***MLH1*[2] *and MSH2*[2]**	1993 and 1999 [220,261]	Candidate gene	Mixed	15 OC cases from Lynch/HNPCC families	Lynch/HNPCC families with at least one OC case (unknown *BRCA1* or *BRCA2* PVs status)	Targeted sequencing of coding-region and splicing junctions of the gene
***MSH6*[2]**	2010 [262]	Candidate gene	Mixed	113 Lynch/HNPCC families positive for pathogenic variants in *MSH6*	Same as above	Whole sequencing of coding-region and splicing junctions of the gene
***PMS2*[2]**	2015 [263]	Candidate gene	Mixed	98 Lynch/HNPCC families positive for pathogenic variants in *PMS2*	Same as above	Same as above

EPCAM	2009 [264]	MSH2 expression	Mixed	10 Lynch/HNPCC families, deficient for MSH2 and exhibit MSI	Same as above	Targeted sequencing of flanking regions of MSH2
RAD51C	2010 [231]	Candidate gene	German	1,100 BC and OC cases from HBC and/or HBOC families and population-matched controls	HBC families with at least two or more cases with BC or HBOC families with at least one or more cases with BC and one with OC, all within first- or second-degree relatives (*BRCA1* or *BRCA2* PV-negative)	Whole sequencing of coding-region and splicing junctions of the gene
RAD51D	2011 [232]	Candidate gene	Mixed	911 BC and OC cases from HBC and/or HBOC families and controls	Same above	Same above
BRIP1	2006 [233]	Candidate gene	Mixed	1,212 BC cases from HBC and HBOC families and controls	HBC or HBOC families with at least two or more cases with BC within first- or second-degree relatives (*BRCA1* or *BRCA2* PV-negative)	Same above
	2011 [234]	Case-control association	Icelandic	656 and 144 sporadic OC cases and population-matched controls	Cases not selected for family history of cancer (Regardless of *BRCA1* or *BRCA2* PVs status)	Whole genome sequencing and genotyping SNP array
***ATM*[2]**	2006 [245]	Candidate gene	Mixed	443 BC from HBC families and controls	Families with at least three or more cases with BC within first- or second-degree relatives, where the index case diagnosed at age of 45 or younger (*BRCA1* or *BRCA2* PV-negative)	Whole sequencing of coding-region and splicing junctions of the gene

	2017 [256]	Candidate gene	Mixed	5,020 sporadic OC cases and controls	OC cases not selected for family history of cancer (Regardless of *BRCA1* or *BRCA2* PVs status)	Same above
PALB2	2007 [244]	Candidate gene	Finnish	113 BC and OC from HBC and HBOC families, 1,918 BC sporadic cases controls	HBC families with at least two or more cases with BC or HBOC families with at least one or more cases with BC and one with OC, all within first- or second-degree relatives (*BRCA1* or *BRCA2* PV-negative) and BC cases not selected for family history of cancer (Regardless of *BRCA1* or *BRCA2* PVs status)	Same above
	2021 [255]	Candidate genes	Mixed	6,385 and 141,135 sporadic OC cases and controls	OC cases not selected for family history of cancer (Regardless of *BRCA1* or *BRCA2* PVs status)	Same above

Information was adapted and modified from reference [182]. [1] As stated in the study by the author. [2] Genes were identified in the context of the associated syndromes, not in breast or ovarian cancers. BC: Breast Cancer; HBC: Hereditary Breast Cancer; HBOC: Hereditary Breast and Ovarian Cancer; HNPCC: Hereditary Non-Polyposis Colorectal Cancer; MSI: Microsatellite Instability; and OC: Ovarian cancer.

Table 1.5.3. The genomic and biological characteristics of ovarian cancer predisposing genes.

OC risk gene	Cytoband	Number of transcripts	Canonical transcript	Size of the canonical transcript in base pairs (bp)	Size of the canonical encoded protein in amino acids (aa)	-/- Mouse phenotype (Reference)
BRCA1	17q21.31	6	NM_007294.4	7,088 bp	1,863 aa	Embryonic lethal [265]
BRCA2	13q13.1	1	NM_000059.4	11,954 bp	3,418 aa	Embryonic lethal [266]
MLH1	3p22.2	23	NM_000249.4	2,494 bp	756 aa	Developed gastrointestinal tumours [267]
MSH2	2p21-p16.3	5	NM_000251.3	3,115 bp	934 aa	Developed lymphoid tumours [268]
MSH6	2p16.3	5	NM_000179.3	4,265 bp	1,360 aa	Developed gastrointestinal and lymphoid tumours [269]
PMS2	7p22.1	15	NM_000535.7	5,093 bp	862 aa	Viable but infertile [270]
EPCAM	2p21	5	NM_002354.3	1547 bp	314 aa	Neonatal lethal with pathological features, including epithelial tufts, enterocyte crowding, altered desmosomes and intercellular gaps [271]
RAD51C	17q22	5	NM_058216.3	2,562 bp	376 aa	Embryonic lethal [272]
RAD51D	17q12	5	NM_002878.4	9,966 bp	328 aa	Embryonic lethal [273]
BRIP1	17q23.2	5	NM_032043.3	8,182 bp	1,249 aa	Viable but sub-fertile [274]
ATM	11q22.3	5	NM_000051.4	12,915 bp	3,056 aa	Proportional lethality but all radiosensitive [275]
PALB2	16p12.2	5	NM_024675.4	4,008 bp	1,186 aa	Embryonic lethal [276]

Information was summarized from different databases (tark.ensembl.org/web/manelist/), (omim.org), (genome.ucsc.edu) and (ncbi.nlm.nih.gov/gene).

Table 1.5.4. The contribution of carriers of germline pathogenic variants in known ovarian cancer risk genes and age at diagnosis with the disease.

OC risk gene	Average age at diagnosis with OC (Reference)	Contribution (%) of familial or sporadic OC carriers of germline pathogenic variants		OC histopathological subtype in the context of the identified germline pathogenic variants (reference)
		Families (Family history of the specified cancer)	Sporadic cases (Reference)	
BRCA1	51 years [206]	17.7% [251] (at least 1 OC and 2 BC cases) 25.7% [251] (at least 2 OC cases regardless of BC)	3.8% [277]	Mainly HGSC followed by EC, CCC and then LGSC [100]
BRCA2	61 years [206]	10.6% [251] (at least 1 OC and 2 BC cases) 17.1% [251] (at least 2 OC cases regardless of BC)	4.2% [277]	Mainly HGSC followed by EC, CCC and then LGSC [100]
MLH1	45 years [278]	0.1% [224] (at least 1 OC and 1 BC cases) 3.5% [278] (at least 1 CC and 1 ENC cases)	0.04% [277]	Mainly CCC or EC [109]
MSH2	43 years [278]	0.5% [224] (at least 1 OC and 1 BC cases) 5.4% [278] (at least 1 CC and 1 ENC cases)	0.1% [277]	Mainly CCC or EC [109]
MSH6	46 years [278]	0.5% [224] (at least 1 OC and 1 BC cases) 3.8% [251] (at least 1 CC and 1 ENC cases)	0.4% [277]	Mainly EC or CCC followed by HGSC [109]
PMS2	52 years [279]	0.4% [279] (at least 1 OC and 1 BC cases) 2.2% [224] (at least 1 CC and 1 ENC cases)	0.04% [277]	Mainly EC or CCC [109]
RAD51C	62 years [239]	1.3% [231] (at least 1 OC and 2 BC cases) 5.7% [251] (at least 2 OC cases regardless of BC)	0.6% [240]	Mainly HGSC followed by EC, CCC and LGSC [100]
RAD51D	57 years [239]	0.9% [232] (at least 1 OC and 2 BC cases) 1.7% [232] (at least 2 OC cases regardless of BC)	0.4% [240]	Mainly HGSC followed by EC, CCC and LGSC [100]
BRIP1	65 years [239]	2.1% [280] (at least 1 OC and 2 BC cases) 4.2% [280] (at least 2 OC cases regardless of BC)	0.9% [240]	Mainly HGSC followed by EC and LGSC [100]

ATM	Not specified	0.5% [257] (at least 1 OC and 2 BC cases)	0.9% [256]	Mainly HGSC [100]
PALB2	Not specified	0.9% [257] (at least 1 OC and 2 BC cases)	0.4% [255]	Mainly HGSC followed by CCC and LGSC [100]

Associated risks are based on the National Comprehensive Cancer Network® (NCCN) Clinical Practice Guidelines in Oncology - guidelines of the Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic – Version 2.2022 (nccn.org/guidelines/category_2). BC: Breast Cancer; OC: Ovarian Cancer; HGSC: High-Grade Serous Carcinoma; LGSC: Low-Grade Serous Carcinoma; EC: Endometrioid Carcinoma; CCC: Clear Cell Carcinoma; MC: Mucinous Carcinoma; CC: Colorectal Cancer; and ENC: Endometrial Cancer.

Table 1.5.5. The associated hereditary cancer syndromes with ovarian cancer predisposing genes.

Ovarian cancer risk gene	OMIM ID[1]	Established associated hereditary cancer syndromes and other non-cancer syndromes (Mode of inheritance)[1,2]	Other associated cancers (Mode of inheritance and reference)
BRCA1	113705	- HBC syndrome, type 1 (AD) - HBOC syndrome, type 1 (AD) - Pancreatic cancer (AD) - Prostate cancer (AD)	- Biliary tract cancer (AD) [281] - Gastric cancer (AD) [281,282] - Male breast cancer (AD) [282]
		- Fanconi anemia, complementation group S (AR)	-
BRCA2	600185	- HBC syndrome, type 2 (AD) - HBOC syndrome, type 2 (AD) - Pancreatic cancer (AD) - Prostate cancer (AD) - Melanoma (AD) - Wilms tumour (AD)	- Esophageal cancer (AD) [281] - Gastric cancer (AD) [281] - Male breast cancer (AD) [281,282]
		- Fanconi anemia, complementation group D1 (AR)	-
MLH1	120436	- Lynch/HNPCC syndrome, type 2 (AD) - Endometrial cancer (AD) - Muir-Torre syndrome (AD) - Ovarian cancer – LS/ HNPCC syndrome (AD)	-
		- Mismatch repair cancer syndrome, type 1 (AR)	-
MSH2	609309	- Lynch/HNPCC syndrome, type 1 (AD) - Endometrial cancer (AD) - Muir-Torre syndrome (AD) - Ovarian cancer – LS/ HNPCC syndrome (AD)	-

		- Mismatch repair cancer syndrome, type 2 (AR)	-	
MSH6	600678	- Lynch/HNPCC syndrome, type 5 (AD) - Endometrial cancer (AD) - Ovarian cancer – LS/ HNPCC syndrome (AD)	-	
		- Mismatch repair cancer syndrome, type 3 (AR)	-	
PMS2	600259	- Lynch/HNPCC syndrome, type 4 (AD) - Ovarian cancer – LS/ HNPCC syndrome (AD)	-	
		- Mismatch repair cancer syndrome, type 4 (AR)	-	
EPCAM	185535	- Lynch/HNPCC syndrome, type 8 (AD) - Endometrial cancer (AD) - Ovarian cancer – LS/HNPCC syndrome (AD)	-	
		- Congenital tufting enteropathy (AR)	-	
RAD51C	602774	- Ovarian cancer (AD)	-	Breast cancer (AD) [283,284]
		- Fanconi anemia, complementation group O (AR)	-	
RAD51D	602954	- Ovarian cancer (AD)	-	Breast cancer (AD) [283,284]
BRIP1	605882	- Ovarian cancer (AD)	-	
		- Fanconi anemia, complementation group J (AR)	-	
ATM	607585	- Breast cancer (AD) - Pancreatic cancer (AD) - Ovarian cancer (AD)	-	
		- Ataxia-telangiectasia (AR)	-	
PALB2	610355	- Breast cancer (AD) - Pancreatic cancer (AD) - Ovarian cancer (AD)	-	Male breast cancer (AD) [285]
		- Fanconi anemia, complementation group N (AR)	-	

[1] Information was summarized from (omim.org). [2] Based on the National Comprehensive Cancer Network® (NCCN) Clinical Practice Guidelines in Oncology – guidelines of the Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic – Version 2.2022 and NCCN® Clinical Practice Guidelines in Oncology – guidelines of the Genetic/Familial High-Risk Assessment: Colorectal – Version 1.2022 (nccn.org/guidelines/category_2). AD: Autosomal Dominant inheritance; AR: Autosomal Recessive inheritance; HBC: Hereditary Breast Cancer Syndrome; HBOC; Hereditary Breast and Ovarian Cancer Syndrome; HNPCC: Hereditary Non-Polyposis Colorectal Cancer Syndrome; and LS: Lynch Syndrome.

Table 1.5.6. Summary of ovarian and/or breast cancer risks and current recommendations for clinical management per ovarian cancer risk gene.

OC risk gene	Absolute risk for OC	Level of evidence of the increased risk for OC (Quality)	Recommendation for OC clinical management (Age group)	Absolute risk for BC	Level of evidence of the increased risk for OC (Quality)	Recommendation for BC clinical management (Age group)
BRCA1	39-58%	Very strong (1)	RRSO (35-40 years)	>60%	Very strong (1)	Every 6-12 months, breast MRI with contrast (>25 years) and mammogram (>30 years); RRM (35-40 years)
BRCA2	13-29%	Very strong (1)	RRSO (40-45 years)	>60%	Very strong (1)	Every 6-12 months, breast MRI with contrast (>25 years) and mammogram (>30 years); RRM (40-45 years)
MLH1	>10%	Strong (2)	Consider RRSO and hysterectomy (35-40 years)	<15%	Limited (3)	None
MSH2	>10%	Strong (2)	Consider RRSO and hysterectomy (35-40 years)	<15%	Limited (3)	None
MSH6	<14%	Strong (2)	Consider HYS; Insufficient evidence for RRSO but potentially beneficial	<15%	Limited (3)	None
PMS2	<3%	Limited (2)	Consider HYS; Insufficient evidence for RRSO and potentially not beneficial	<15%	Limited (3)	None

EPCAM	<10%	Limited (2,3)	Consider HYS; Insufficient evidence for RRSO	<15%	Limited (3)	None
RAD51C	>10%	Strong (2,3)	Consider RRSO (40-50 years)	15-40%	Limited (2,3) but potential increased risk	Insufficient evidence for RRM
RAD51D	>10%	Strong (2,3)	Consider RRSO (40-50 years)	15-40%	Limited (2,3) but potential increased risk	Insufficient evidence for RRM
BRIP1	>10%	Strong (2,3)	Consider RRSO (40-50 years)	Unknown	Limited (3) but potential increased risk	None
ATM	<3%	Strong (3)	Insufficient evidence for RRSO and controversial benefits	15-40%	Strong (2,3)	Annual mammogram or consider MRI with contrast (>40 years); Insufficient evidence for RRM
PALB2	3-5%	Strong (2,3)	Insufficient evidence for RRSO	41-60%	Strong (2)	Annual mammogram or consider MRI with contrast (>30 years); Consider RRM

Information was adapted and modified from references [10,99,182,286,287] and the Oxford Center for Evidence-Based Medicine: Levels of Evidence – March 2009 (cebm.ox.ac.uk). Associated information in breast cancer (BC) was for comparison purposes. [1] All associated risks are based on the National Comprehensive Cancer Network® (NCCN) Clinical Practice Guidelines in Oncology - guidelines of the Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic – Version 2.2022 (nccn.org/guidelines/category_2). BC: Breast Cancer; MRI: Magnetic Resonance Imaging; OC: Ovarian cancer; HYS: Hysterectomy; RRM: Risk-Reducing Mastectomy; and RRSO: Risk-Reducing Salpingo-Oophorectomy.

RRSO for *ATM* or *PALB2* PVs carriers as a standard of practice, yet it can be discussed after consideration of the family history of cancer and the associated personal risks and benefits; if the procedure of RRSO is opted for, it can be deferred until the age of menopause [99,101].

Other BC risk genes such as *BARD1* [283,284] and *CHEK2* [288] have been proposed as candidates for OC predisposition [100], yet there has been a general lack of evidence to support their role in OC risk [99–101,156,184,289]. Both genes were identified as BC risk genes using a candidate gene approach and are also involved in the DNA repair pathway; the applied approaches that facilitated these findings was recently reviewed by Dr. Patricia Tonin's group [182,289]. BARD1 is a binding partner of BRCA1 and assists in stabilizing the double-strand DNA break site for repair by the HR pathway, whereas CHEK2 is a cell cycle checkpoint regulator upstream of FA and HR pathways [151]. Based on the current clinical management guidelines, there is no recommendation for RRSO for carriers of PVs in either of these genes [99].

1.6. The missing heritability for ovarian cancer and major challenges in the discovery of new ovarian cancer predisposing genes

Not long after the discovery of *BRCA1* [180] and *BRCA2* [181] as OC and BC risk genes, it was clear that not all HBOC families could be explained by PVs in either of these genes [182,290,291]. The proportion of OC families negative for PVs in *BRCA1* or *BRCA2* is approximately 55%, ranging between 5-65% depending on the population studied [100,189,190]. This directed some research groups to investigate for new OCPGs that could explain the remaining cases [168,179]. Yet, this process of identifying new OCPGs has been challenging may be due to the fact that: (1) OC families negative for PVs in *BRCA1* and *BRCA2* are more genetically heterogeneous than previously expected [290,292–295]; or (2) another single major risk gene that could explain the remaining *BRCA1* and *BRCA2* PV-negative OC families is unlikely [182,296]. In other words, the contribution of new moderate- to high-risk genes to hereditary OC could be considerably lower relative to *BRCA1* and *BRCA2* [168,182,296]. This resulted in a debate in the research community regarding the merit of pursuing new risk genes, given that *BRCA1* and *BRCA2*-negative OC families could also be due to chance clustering of

sporadic OC cases [182,295,297]. By early 2010, three main discoveries involving new OCPGs were reported: *BRIP1* [234], *RAD51C* [231] and *RAD51D* [232] as discussed in **sub-section 1.5.2.2**. The combined contribution of PVs in these genes is, indeed, small of less than 5% in OC families and 2% in OC cases not selected for family history of cancer [100,242,257,298]. Altogether, the genetic heterogeneity of OC families negatives for *BRCA1* and *BRCA2* PVs and the paucity of new candidate OC risk genes have created major challenges in the identification of new OCPGs.

1.6.1. Approaches and methods to facilitate the identification of new moderate-to-high ovarian cancer risk genes

The identification of new candidates for OC predisposition is dependent on the approach and the method being applied [182,299,300].

This process in identifying new candidates OCPGs can be facilitated by the investigating families enriched with OC cases in comparison to OC cases not selected for family history of cancer as the likelihood of identifying PVs that are associated with the disease is increased in these families [109,301]. A trend towards a higher frequency of carriers of candidate risk variants in familial cancer cases versus in sporadic cancer cases than that in cancer-free controls is well-documented in known CPGs [100,165,182], including *RAD51C* [231] and *RAD51D* [232]. Therefore, observing such a trend is usually the first step in supporting the candidacy of a new cancer risk gene [182,299]. However, the paucity of families with two or more closely related OC cases who are confirmed to be negative for PVs in *BRCA1* or *BRCA2* is an obstacle in the discovery of new OCPGs [182]. The proportion of families with two first-degree relatives with OC is estimated to be less than 5%, and less than 1% for families with more than two first-degree relatives with OC, regardless of *BRCA1* or *BRCA2* carrier status [150,281,302]. Over the past few years, national and international consortia have been developed to increase the pool of familial cancer cases that are suitable for identifying and validating new OC risk genes [182] such as *RAD51C* [231] and *RAD51D* [232].

While the importance of family-based studies for the discovery of new OCPGs is apparent, the considerable amount of effort and time involved in recruiting families has led to case-control studies for such discoveries [182,300,303]. Large-scale, well-

powered studies of cases and controls has facilitated the identification of new CPGs when a particular PV or PVs in a gene achieve a statistical enrichment in cases versus controls, after adjusting for multiple testing [300]. This supports the candidacy of a new cancer risk gene [299,300] such as *BRIP1* [234]. This approach of using cases and controls are now feasible due to the dramatic advancement in targeted gene-panel or next-generation sequencing technologies [299,300]. However, there are two main critical issues: (1) the controls that are selected should be population-matched with the cancer cases; and (2) the contribution of a new candidate CPG should be validated in independently ascertained cancer cases from the same population and/or from other populations [182].

An additional criterion to consider when selecting cancer cases is the age at diagnosis of cancer, the rationale being that developing cancer at an earlier age could be an indicator of an underlying inherited predisposition to cancer [34,112]. Hence, selecting cases with younger age at diagnosis is one strategy to increase the likelihood of identifying new CPGs [182]. These younger cases can be selected for family history of cancer or not selected for family history of cancer. In the context of hereditary OC, the median age at diagnosis with the disease is in the early-60s, and the proportion of women who developed epithelial OC before the age of 50 years is approximately 30% [2,3]. Indeed, the selection of cancer cases based on family history of cancer and age at diagnosis should be carefully considered when attempting to identify new cancer risk genes as validation is required in all age groups [34].

In addition to the different approaches of the study design and type of cancer cases in the identification of new candidate CPGs, one should also evaluate the best option in terms of the methodology and analyses that can been used [179,182,300,303]. Classical genome-wide linkage analysis has been the most successful approach, yielding the discovery of over 50 CPGs [179], including *BRCA1* [180] and *BRCA2* [181]. This strategy allows successful tracking of potential disease-associated genomic markers in high penetrance familial cancer clusters in large multi-generational cancer families [179,182,292,304]. However, the paucity of multi-generational families and the advances in next-generation sequencing has led to a shift in strategy [179,182]. The candidate gene approach has since been adopted by many research groups and has

shown promise in the discovery of CPGs [179]. Applying this approach relies on the current knowledge of the biological function of the gene; the rationale being that germline PVs in protein-encoding gene(s) that are in direct interaction or in the same complex with, or are involved in the same pathway as a known risk gene may be associated with a similar phenotype [179,182,303]. For example, in the context of hereditary OC, advances in understanding the biology of BRCA1 and BRCA2 as key players in specific DNA repair pathways (described in **sub-section 1.5.2.2.**) appear to favour genes involved in various DNA repair pathways as a candidate gene approach [182]; general characteristics of DNA repair pathways and known and candidate CPGs are presented in **Table 1.6.1.** and **Figure 1.6.** Indeed, this approach facilitated the discovery of *RAD51C* [231] and *RAD51D* [232] are involved in the repair of double-strand DNA breaks by the HR pathway as described in **sub-section 1.5.2.2.**, along with BRCA1 and BRCA2 (**Figure 1.6.**) [151]. Despite the investigation of a large number of candidate CPGs, the majority of these have not been supported yet [179]. On the other hand, using the genome-wide association approach has identified moderate-to-high cancer risk genes, though they are very few [179]. This latter approach can be applied to genotyping- or sequencing-based data by selecting for a PV or several PVs that are shown to be statistically enriched in cancer cases versus controls [303,305] such as *BRIP1* [234]. Although the genome-wide association approach has proven successful in identifying new CPGs in an unbiased way, this approach requires thousands of cancer cases and controls [160,305]. In summary, there is no gold standard approach for identifying new CPGs as each approach has its advantages and disadvantages, and a combination of two or more strategies may facilitate discovery.

As I mentioned in **section 1.6.**, the rarity of carriers of PVs in new OCPGs and the genetic heterogeneity of hereditary OC cases known to be negative for PVs in *BRCA1* and *BRCA2* explain the difficulty in identifying new candidate genes for OC predisposition. Although the abovementioned approaches have been shown to successfully identify new cancer risk genes, large sample sizes of cancer cases are required. For example, over 900 HBOC families of mainly of Western European origin were investigated to identify *RAD51C* and *RAD51D* [231,232]. One way to overcome issue of sample size is to investigate a founder population exhibiting a unique genetic

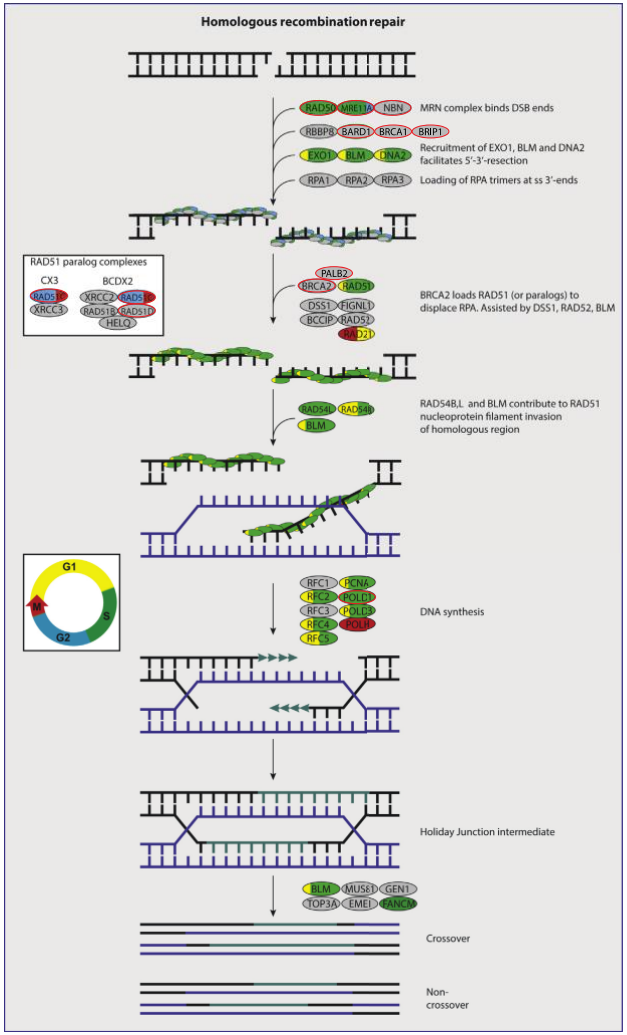
Table 1.6.1. Overview of the core DNA repair pathways with their types of DNA damage and stimuli.

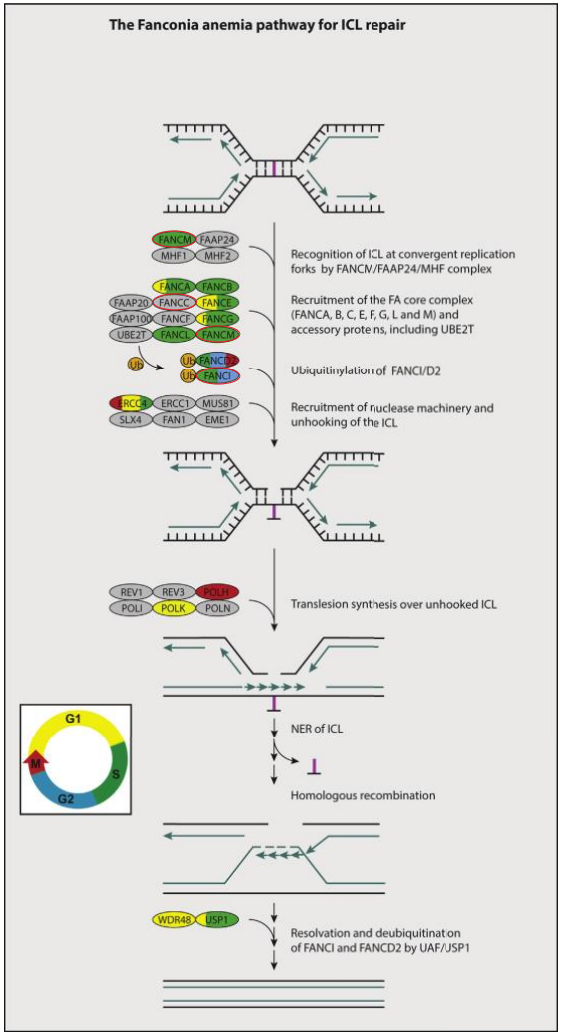
	Double-stranded DNA Repair			**Single-stranded DNA Repair**			**Others**
Core DNA repair pathways	HR	FA	NHEJ (Canonical or alternative)	BER (Direct or indirect)	NER	MMR	TLS
DNA damage stimulus/agent	External: Irradiation; Topoisomerase I/II inhibitors; Replication inhibitors Internal: Unrepaired Single strand DNA breaks; Reactive oxygen species			External: Irradiation; Alkylating agent; Topoisomerase I inhibitors; Ultraviolet Internal: Reactive oxygen species; Base deamination	External: Ultraviolet; Alkylating agent; Tobacco; Aflatoxin Internal: Reactive oxygen species	Internal: Replication errors; Base deamination	External: Smoking; Ultraviolet
Type of DNA damage (Estimated number of DNA damage per cell cycle)	Double strand DNA breaks; Stalled Replication forks (10-50)			Base depyrimidination (500-10,000); Base depurination (200-100,000); Base deamination, oxidation or alkylated (100-500); Single strand DNA breaks (50,000)	Single strand DNA breaks (50,000)	Base mismatched (10,000-100,000)	Bulky DNA lesions, including adducts (vary)

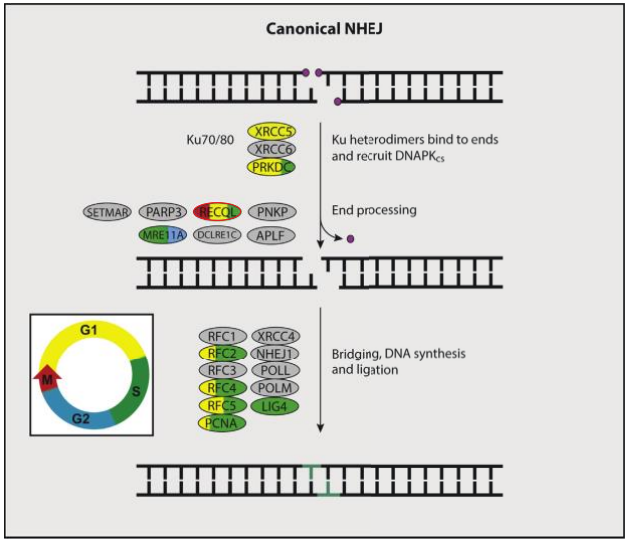
Information was summarized from references [306–312].

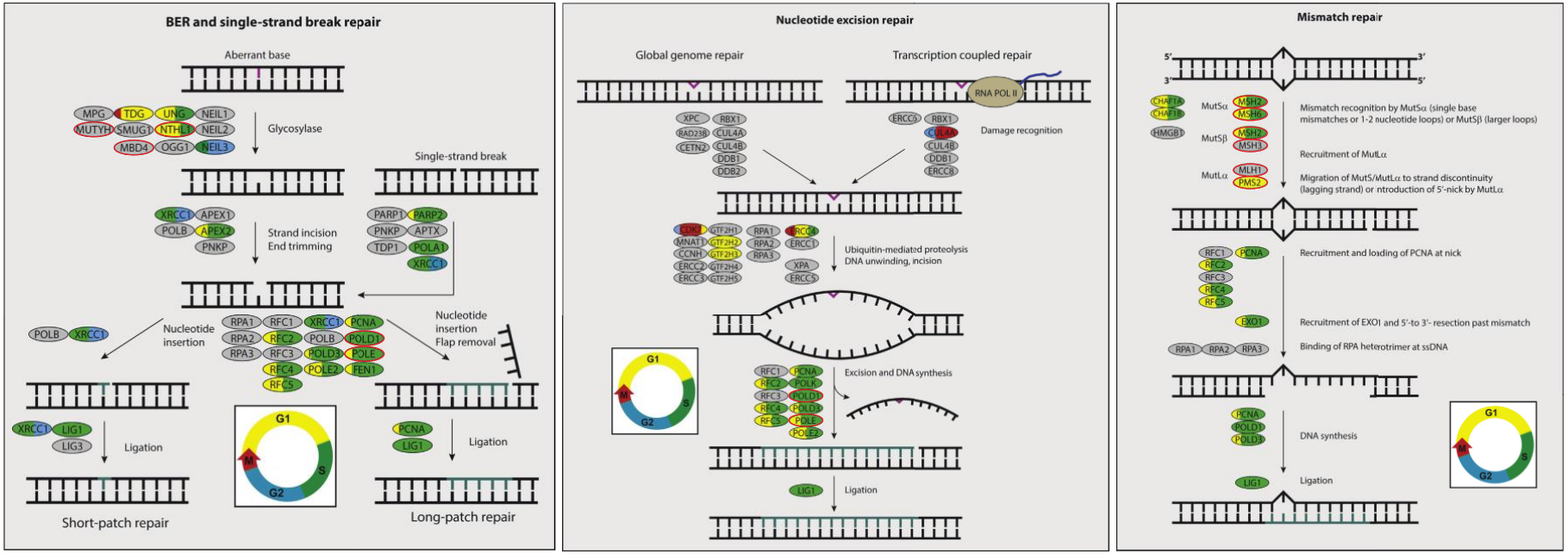
BER: Base Excision Repair; FA: Fanconi anemia; HR: Homologous Recombination; NER: Nucleotide Excision Repair; NHEJ: Non-Homologous End Joining; MMR: Mismatch Repair; and TLS: Translation DNA synthesis.

Figure 1.6.1. Overview of the core DNA repair pathways during the cell cycle.









This figure was adapted and modified from [311], which were published in the journal DNA Repair and licensed under a Creative Commons Attribution 3.0 License (creativecommons.org/licenses/by/3.0/). Known and newly identified cancer predisposing genes were circled in red.

architecture due to genetic drift [182]. In the following sub-section, I summarized the concept of genetic drift and founder effects, focusing on the French Canadian (FC) population of the province of Quebec, Canada. Dr. Patricia Tonin's lab has been studying the FCs of Quebec as a model to characterize known CPGs and to identify new candidate risk genes for OC and/or BC. I then presented the merits of genetically characterizing known CPGs or identifying new candidate risk genes for OC in the FCs.

1.6.2. Genetic drift and the founder effect

Genetic drift is described as a random change in the frequency of a genetic variant, resulting in a significant reduction in the genetic variation in a small population relative to its original population [313,314]. This phenomenon could occur by founder effects when a small group of individuals, so-called founders, have become isolated due to geographical, cultural, religious or other reasons from the original population and continue to expand, resulting in changes in allele frequencies compared to the original population and reduced genetic diversity [313–315]. This effect is observed as a significant increase (or decrease) in the frequency of carriers of specific rare disease-associated variants who inherited the variant from a common ancestor (cancer.gov/publications/dictionaries/genetics-dictionary) [315]. Therefore, investigating founder populations to identify new CPGs can be justified by the fact that a PV could attain a disproportionately higher frequency by genetic drift than in the general population [182,315]. An example of attaining a high frequency of a rare PV in the general population is one particular variant in *BRIP1* that was reported to account for 2.4% of 318 OC cases not selected for family history of cancer in the Icelandic population [234]. By contrast, 0.82% of 3,429 sporadic OC cases from the Western European general population carried this variant [33]. There are other founder populations, in addition to the Icelandic population [316], that have been well-recognized for their contribution to research on cancer genetics such as the Ashkenazi Jewish population of Eastern European ancestry [317], the Finnish population [318] and the FC population of the province of Quebec, Canada [182,319,320]. Indeed, investigating a small number of cancer cases from the Icelandic founder population led to the discovery of *BRIP1* [234] while the study of the Finnish population and the FC population of

Quebec led to the discoveries of *PALB2* [244] and *RECQL* [321] as new BC risk genes. There are other populations exhibiting unique genetic architectures, yet they are under-investigated (see **Appendix I**).

The history of the FCs as a founder population begins with approximately 2,600 French individuals who settled in regions along the St. Lawrence River in 1608 [322]. After immigration from France ceased with the British Conquest in 1759, the Acadians, who were descendants of the original French settlers that settled in Acadia (the current provinces of Nova Scotia, New Brunswick and Prince-Edward Island), moved to several different regions in Quebec to escape deportation by the British [323,324]. By the end of the 1700s, the American Loyalists, who wanted to remain under the British rule, also moved to Quebec [323,325]. Meanwhile, the FC population expanded rapidly in relative isolation due a high fertility rate that facilitates genetic drift. As the population grew in size, the settlers colonized new regions of Quebec, including remote and isolated regions, which resulted in the creation of subpopulations [326–328]. The settlement in the Saguenay-Lac-St-Jean region began around 1840 with settlers from the neighboring region of Charlevoix, and by 1910, approximately 75% of the estimated 30,000 immigrants to Saguenay-Lac-St-Jean were from Charlevoix [322,327]. The region of the North Shore of the St. Laurence River was mainly colonized by people from the Charlevoix and Bas-St-Laurent regions between 1840 and 1920 [322,327,329]. The region of Gaspésie on the south side of the St. Laurence River, was colonized by Europeans, Acadians, American Loyalists, and FCs; all these groups then evolved relatively separately as they predominantly married within their subpopulation [322,323,327]. During the 1800s and 1900s, immigrants from various origins married into the FC population with a very limited genetic impact, whereas it has been shown that the early founders made a greater contribution to the current FC gene pool [326,330].

The availability of genealogical data dating back over 400 years has greatly facilitated genetic research in the contemporary FC population [323]. This data is mainly based on birth, marriage and/or death certificates that cover the province of Quebec from the 1600s [323,331,332]. It has been estimated that the majority of the present-day FC population of eight million are descendants of approximately 10,000 Western

Europeans, including about 8,500 of the original French settlers in 1608 [322,327]. Further genetic analyses showed that the gene pool of the FC population of Quebec exhibits a reduced level of diversity, yet not significantly different from that of Western European populations, including France [333,334]. For example, it was found that there are some mutually exclusive haplotypes between the FC population of Quebec and the population of France [334]. In addition, it was shown that there is an excess of potentially deleterious unique and/or rare germline variants identified by WES in the FC population of Quebec versus the population of France [333]. This is mainly attributed to the number of founders and their diversity as well as to the degree of their isolation and the magnitude of the growth rate and marriage predominantly within subpopulations [334]. It was also shown that there are regional founder effects [323,326,328,333–336]. For example, the pattern of sharing identical-by-descent, which is defined as identical chromosomal segments that are shared between two individuals who inherited the segment from a common ancestor, was different across the province of Quebec, but remained consistent with the settlement history [322,323]. This can be explained by the successive migrations of settlers from the original founders of the initial sites of settlement [327,334]. These analyses also showed regional founder effects among the FCs of Quebec, Acadians and the Loyalists [326,334]. Nonetheless, the more recent regional founder effects have a limited impact on the gene pool of the contemporary FCs, with the early founders having a greater contribution of at least 90% of the regions in the province of Quebec [326].

In the context of hereditary OC, the genetic heterogeneity in the FC population of Quebec is reflected in the wide spectrum of approximately 40 PVs in *BRCA1* or *BRCA2*, associated with different carrier frequencies, relative to those in the other well-studied founder populations [182,319,320,334]. By contrast, only three PVs in *BRCA1* or *BRCA2* were reported to account for up to 75% of OC families in the Ashkenazi Jewish population, and only one *BRCA2* PV accounts for up to 70% of OC families in the Icelandic population [337,338]. The most frequently occurring PVs in known OCPGs and their contribution to OC families and sporadic cases in well-characterized founder populations, including the FC population of Quebec, are summarized in **Table 1.6.2.** The spectrum of these variants is limited relative to over 5,000 PVs in *BRCA1* or *BRCA2*

identified in Western European populations [338–341]. This diversity is also reflected in PVs in *RAD51D* [342] and *PALB2* that increase risk for hereditary OC and BC, respectively [182]. Haplotype analyses of the chromosomal segments containing the most recurrent PVs in *BRCA1* or *BRCA2* in FC carriers, suggests that carriers of each of these recurrent PVs are likely to share a common ancestor as shown in **Table 1.6.2.** The usage of the term 'recurrent PV' here refers to the identification of the same PV in families or cases not known to be related, that is: (1) the mutational event giving rise to the PV occurred independently more than once; or (2) the PV was inherited from a common ancestor, but genealogical data was unavailable to link the families or cases. Five of the recurring *BRCA1* or *BRCA2* PVs were found to account for approximately 40% of the OC families [344] and 6.6% of the sporadic cases [345]. In a recent report, 5.4% of 222 OC cases, who were referred from different regions of Quebec for gene-panel testing, were found to harbour five *BRCA1* or *BRCA2* PVs, and four of which were the known founder variants [346]. FC carriers of the most recurrent *BRCA1* and *BRCA2* PVs, who share identical haplotypes, are predominantly distributed in the regions surrounding the St. Lawrence River [347]. This is due to the pattern of settlement of the original founders in 1608 [322], suggesting that at least two (marked with [2] in **Table 1.6.2.**) of these variants are likely to have been introduced early in the settlement of Quebec [347]. Genealogical data linked the most recurrent *BRCA1* PV c.4327C>T; p.Arg1443Ter back to a couple from France and Portugal that were married in 1761 in Quebec [182]. It was also shown that carriers of this recurrent *BRCA1* PV (**Table 1.6.3.**) were identified in the Saguenay-Lac-St-Jean region [347], supporting the hypothesis of waves of expansion of the FCs after 1840 [322]. The frequency and geographical distribution of carriers of these recurrent variants are consistent with the recent report [346]. It is clear that the founders of recurrent mutations did not contribute uniformly to the contemporary gene pool of the FCs of Quebec, resulting in the observed genetic architecture.

Table 1.6.2. The most frequently occurring germline pathogenic variants in known ovarian cancer predisposing genes and their contribution in families and cases in the French Canadian and other founder populations.

	BRCA1	***BRCA2***	***MLH1*, *MSH2*, *MSH6* or *PMS2***	***BRIP1, RAD51C* or *RAD51D***	***PALB2* or *ATM***
French Canadians of Quebec	- c.962G>A; p.Trp321Ter - c.1054G>T; p.Glu352Ter - c.1961dup; p.Tyr655ValfsTer18 - c.2125_2126ins; p.Phe709Tyrf - **c.2834_2836delins; p.Ser945ThrfsTer6** - **c.3649_3650ins; p.Ser1217TyrfsTer2** - c.3756_3759del; p.Ser1253ArgfsTer10 - c.4041_4042del; p.Gly1348AsnfsTer7 - **c.4327C>T; p.Arg1443Ter[1,2]** - c.5102_5103del; p.Leu1701GlnfsTer14	- **c.2588dup; p.Asn863LysfsTer18** - **c.2808_2811del; p.Ala938ProfsTer21** - **c.3170_3174del; p.Lys1057ThrfsTer8** - c.3545_3546del; p.Phe1182Ter - **c.5857G>T; p.Glu1953Ter** - **c.6275_6276del; p.Leu2092ProfsTer7** - **c.8537_8538del; p.Glu2846GlyfsTer22[2]** - c.9004G>A; p.Glu3002Lys	- ***MLH1* c.2195_2198dup; p.His733GlnfsTer14** - ***MLH1* c.1039-2329_1409+827del; p.Thr347_LysfsTer8** (deletion of 3.5 kilobase pairs which includes exon 12) - ***MSH6* c.10C>T; p.Gln4Ter**	- ***RAD51D* c.620C>T; p.Ser207Leu**	- ***PALB2* c.2323C>T; p.Gln775Ter**
	Ten *BRCA1* variants combined - 35-40% of familial OC - 10-15% of sporadic OC	Eight *BRCA2* variants combined - 15-20% of familial OC - 5-6% of sporadic OC	Two *MLH1* and one *MSH6* variants combined - Unknown in familial OC - Unknown in sporadic OC	**One *RAD51D* variant** - Unknown in familial OC - 3-4% of sporadic OC	One *PALB2* variant - <1% of familial OC - <1% of sporadic OC
Finnish	- c.2804del; p.Pro935HisfsTer18 - **c.3485del; p.Asp1162ValfsTe**	- c.771_775del; p.Asn257LysfsTer17[1]	- ***MLH1* c.454-1G>A**	- ***RAD51C* c.837+1G>A**	- ***PALB2* c.1592del; p.Leu531CysfsTer30**

	- **c.3626del; p.Leu1209Ter** - **c.4097-2A>G** - c.4096+3A>G - **c.4327C>T; p.Arg1443Ter**[1] - **c.5251C>T; p.Arg1751Ter**	- **c.7480C>T; p.Arg2494Ter** - **c.8327T>G; p.Leu2776Ter** - **c.9118-2A>G** - c.9117+1G>A	- ***MLH1* c.1731+2247_1897-402del; p.Pro579_Glu633del** (deletion of 3.5 kilobase pairs which includes exon 16) - ***MSH6* c.2983G>T; p.Glu995Ter**	- ***RAD51C* c.93del; p.Phe32SerfsTer8** - ***RAD51D* c.576+1G>A**	- ***ATM* c.7570G>C; p.Ala2524Pro and c.6908dup; p.Glu2304GlyfsTer69**
	Seven *BRCA1* variants combined - 15-25% of familial OC - 5% of sporadic OC	Five *BRCA2* variants combined - 5-15% of familial OC - 1% of sporadic OC	Two *MLH1* and one *MSH6* variants combined - Unknown in familial OC - Unknown in sporadic OC	Two *RAD51C* variants combined - 1-2% of familial OC - 1% of sporadic OC 1 *RAD51D* variant - 2-3% of familial OC - <1% of sporadic OC	One *PALB2* variant - <1% of familial OC - <1% of sporadic OC 2 *ATM* variants combined - <1% of familial OC - <1% of sporadic OC
Ashkenazi Jewish	- **c.68_69del; p.Glu23ValfsTer1** - **c.5266dup; p.Gln1756ProfsTer74**	- **c.5946del; p.Ser1982ArgfsTer22**	- ***MSH2* c.1906G>C; p.Ala636Pro** - ***MSH6* c.3984_3987dup; p.Leu1330ValfsTer12** - ***MSH6* c.3959_3962del; p.Ala1320GlufsTer6**	None was reported	None was reported
	Two *BRCA1* variants combined - 40-50% of familial OC - 30-35% of sporadic OC	One *BRCA2* variant combined - 20-25% of familial OC - 15% of sporadic OC	One *MSH2* and Two *MSH6* variants combined - Unknown in familial OC		

			- <1% of sporadic OC		
Icelandic	- **c.5074G>A; p.Asp1692Asn** One *BRCA1* variant - <1% of familial OC - <1% of sporadic OC	- **c.771_775del; p.Asn257LysfsTer17**[1] One *BRCA2* variant - 70% of familial OC - 8-10% of sporadic OC	- ***MSH6* c.1754T>C; p.Leu585Pro** - ***PMS2* c.736_741del6ins11; p.Pro246CysfsTer3** - ***PMS2* c.354-43T>A** One *MSH6* and two *PMS2* variants combined - <1% of familial OC - Unknown in sporadic OC	- *BRIP1* c.2041_2042dup; p.Leu681PhefsTer8 One *BRIP1* variant - Unknown in familial OC - 3% of sporadic OC	None was reported

Information was summarized from references [182,223,337,338,348–350], and contributions in percentages are approximations in the context of hereditary ovarian cancer (OC) cases from families with different phenotypes or those cases not selected for family history of cancer harbouring the frequently occurring pathogenic variants listed above. Shared haplotypes were identified in carriers of variants (in bold) by haplotype analysis and were summarized from references [347,351–367]; carrier frequencies were summarized from references [234,344,345,347,350,356,357,360,368–377]. Annotated variants were based on the human genome reference (GRCh37/hg19): *BRCA1* (NM_007294.4), *BRCA2* (NM_000059.4), *MLH1* (NM_000249.4), *MSH2* (NM_000251.3), *MSH6* (NM_000179.3), *PMS2* (NM_000535.7), *BRIP1* (NM_032043.3), *RAD51C* (NM_058216.3), *RAD51D* (NM_002878.4), *PALB2* (NM_024675.4) and *ATM* (NM_000051.4) which are available at (tark.ensembl.org/web/manelist/). [1] Frequently occurring variants were identified in more than one founder population; and [2] The two most frequently recurring variants in the French Canadian population of Quebec are thought to have been introduced early in the settlement of Quebec in 1608.

Table 1.6.3. General genetic characteristics of selected founder populations.

Founder population	Approximate non-cancer carrier frequency of the most prevalent PV in *BRCA1* or *BRCA2*[1]	Estimated age of the haplotype block with a founder PV in *BRCA1* and/or *BRCA2*[1]	Estimated size of the *BRCA1* or *BRCA2* haplotype block in centimorgan (cM) or megabase pairs (Mb)[1]
French Canadians of Quebec	1 in 1,000 of *BRCA1* c.4327C>T; p.Arg1443Ter	Over 400 years	9.3 cM encompassing *BRCA1* c.4327C>T; p.Arg1443Ter
Finnish	1 in 10,000 of *BRCA1* c.4097-2A>G	150 to 800 years	15 cM encompassing *BRCA1* c.4097-2A>G
Ashkenazi Jewish	1 in 250 of *BRCA1* c.68_69del; p.Glu23ValfsTer17	650 to 1,500 years	2.1 Mb encompassing *BRCA1* c.68_69del; p.Glu23ValfsTer17
Icelandic	1 in 250 of *BRCA2* c.771_775del; p.Asn257LysfsTer17	340 to 1,000 years	1.7 cM encompassing *BRCA2* c.771_775del; p.Asn257LysfsTer17

Information was summarized from references [345,353,355,378–382]. [1] The frequencies of the most common pathogenic variants (PVs) in *BRCA1* and *BRCA2* were obtained from the Genome Aggregation Database (gnomAD) v2.1.1. (gnomad.broadinstitute.org) or summarized from references [337,381].

1.7. The rationale of this thesis project

As mentioned above, it is clear that not all familial OC cases are explained by germline PVs in *BRCA1* and *BRCA2* [182,290]. Before I started my Ph.D. project, it was already reported that approximately 40% of OC families from the FC population of the province of Quebec were negative for PVs in *BRCA1* or *BRCA2* [297,344,347,352,383]. This is consistent with the observation that PVs in known OC risk genes do not account for all hereditary OC families and cases [100,189,190], suggesting other risk genes are yet to be identified.

1.8. Hypothesis

My hypothesis was that women with OC who tested negative for BRCA1 or BRCA2 pathogenic variants harbour moderate-to-high risk variants in other cancer predisposing genes.

1.9. The proposed strategies in this thesis project

In order to test my hypothesis, I proposed three main strategies that were applied in Chapters III, IV and V.

Strategy 1: Investigating the germline of families enriched for OC within the same family branch or OC cases who develop the disease at an early age.

The rationale is that the likelihood of identifying PVs that are associated with the disease is increased in these families and those cases who developed the disease at early onset, as both groups represent phenotypic hallmarks of genetic predisposition, in contrast to cases not selected for age at diagnosis with OC or family history of cancer; such trend of the frequency of a new candidate PV carriers is expected as it has been observed in known cancer risk genes for decades [100,182] as presented in **sub-section 1.6.1.** This approach has already been proven successful in the discovery of *RAD51C* [231] and *RAD51D* [232] as OC risk genes as well as for the discovery of *PALB2* [244], *CHEK2* [288] and *RECQL* [321] as BC risk genes.

Strategy 2: Applying a candidate gene approach by investigating germline variants in genes that play a role in DNA repair pathways.

The rationale for the candidate gene approach is that genes encoding proteins that directly interact with known OCPGs encoding proteins like BRCA1 and BRCA2, or those involved in the same pathway or other DNA repair pathways, may confer risk resulting in the same phenotype [151,179,182] as presented in **sub-section 1.6.1.** This strategy led to the discovery of *RAD51C* [231], *RAD51D* [232] and *FANCI* [384] as OCPGs, and *PALB2* [244] as a BC risk gene.

Strategy 3: Investigating the germline of the FC population of Quebec, a population exhibiting a unique genetic architecture.

The rationale is that a founder population may attain a high frequency of a recurring PV that is rare in the general population, which therefore can be more easily detected in a small number of as cases [182,319,320] as presented in **sub-section 1.6.2.** This approach was responsible for the discovery of *FANCI* [384] a new OC predisposing gene and *RECQL* [321] as a new BC risk gene.

1.10. Main aims

In order to test my hypothesis, I aimed as follows:

Aim 1 (Chapter II): confirm the status of OC families who previously tested negative for PVs in *BRCA1* and *BRCA2*;

Aim 2 (Chapter III): identify new candidate risk variants in *RAD51C* and *RAD51D*; and determine their prevalence in OC cases in the FC population of Quebec;

Aim 3 (Chapter IV): determine the prevalence of candidate risk variants in *BRIP1*, which were reported by adult hereditary cancer clinics, in OC cases in the FC population of Quebec; and

Aim 4 (Chapter V): identify new candidate OC pathogenic variants in genes involved in DNA repair pathways and determine their prevalence in OC cases in FC and non-FC populations.

CHAPTER II: Case Review: Whole-Exome Sequencing Analyses Identify Carriers of a Known Likely Pathogenic Intronic *BRCA1* Variant in Ovarian Cancer Cases Clinically Negative for Pathogenic *BRCA1* and *BRCA2* Variants

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2.1. Preface

With the discovery of *BRCA1* and *BRCA2*, their roles in conferring risk for ovarian and other cancers in various populations have been predominantly investigated by sequencing coding and flanking regions of these genes. Consequently, over 5,000 rare pathogenic variants of these regions of *BRCA1* or *BRCA2* have been reported. As more variants have been deposited in publicly available databases and new computational tools have been developed to predict these variants effects on the transcribed genes, it has been possible to infer their clinical relevance for carriers.

In this chapter, I applied a set of selected in silico tools, that have been shown to have high performance in predicting pathogenicity of a variant, to whole exome sequencing data generated from the germline of index cases with ovarian cancer. These cases were reported to be negative for pathogenic variants in *BRCA1* or *BRCA2* in a medical genetic setting. The main aim of this study was to confirm that these index cases with ovarian cancer were indeed negative for pathogenic variants in *BRCA1* or *BRCA2* for further genetic analyses.

2.2. Abstract

Background: Detecting pathogenic intronic variants resulting in aberrant splicing remains a challenge in routine genetic testing. We describe germline whole-exome sequencing (WES) analyses and apply in silico predictive tools of familial ovarian cancer (OC) cases reported clinically negative for pathogenic *BRCA1* and *BRCA2* variants. **Methods:** WES data from 27 familial OC cases reported clinically negative for pathogenic *BRCA1* and *BRCA2* variants and 53 sporadic early-onset OC cases were analyzed for pathogenic variants in *BRCA1* or *BRCA2*. WES data from carriers of pathogenic *BRCA1* or *BRCA2* variants were analyzed for pathogenic variants in 10 other OC predisposing genes. Loss of heterozygosity analysis was performed on tumor DNA from variant carriers. **Results:** *BRCA1* c.5407-25T>A intronic variant, identified in two affected sisters and one sporadic OC case, is predicted to create a new splice effecting transcription of *BRCA1*. WES data from *BRCA1* c.5407-25T>A carriers showed no evidence of pathogenic variants in other OC predisposing genes. Sequencing the tumor DNA from the variant carrier showed complete loss of the wild-type allele. **Conclusions:** The findings support *BRCA1* c.5407-25T>A as a likely pathogenic variant and highlight the importance of investigating intronic sequences as causal variants in OC families where the involvement of BRCA1 is highly suggestive.

Keywords: familial ovarian cancer; whole exome sequencing; *BRCA1*; germline variant; intronic variant; alternative splicing variant.

2.3. Introduction

The major heritable risk factors for ovarian cancer (OC) are pathogenic germline variants in *BRCA1* [1] or *BRCA2* [2]. Women carrying pathogenic variants in either of these genes are at significantly increased risk for OC from 17% (95% confidence interval [CI]: 11–25%) to 44% (95%CI: 36–53%) by age 80 depending on the gene involved [3], whereas the lifetime risk for OC in the general population is estimated to be 1.2% by age 80 [4,5]. Depending on the population studied, carriers of pathogenic variants in *BRCA1* or *BRCA2* have been identified in 40–85% of OC with a family history of breast cancer (BC) and/or OC (i.e., hereditary breast and ovarian cancer (HBOC) syndrome families) and 10–15% of all epithelial OC [6], regardless of the family

history of cancer.

Over 20,000 variants in *BRCA1* and *BRCA2* identified in the context of hereditary BC and/or OC cases have been reported in the literature or in publicly available databases [7,8]. Approximately 15% of all reported variants have been classified as pathogenic or likely pathogenic based on the American College of Medical Genetics and Genomics (ACMG) guidelines [9], and over 90% of these variants were of the nonsense, frameshift or exon-intron splice junction (±1–2 nucleotides from the exon) type, resulting in a purported loss of gene function [7,10]. A pathogenic or likely pathogenic variant classification is mainly under the assumption that such loss-of-function (LoF) variants are more likely to result in a premature amino acid termination eliciting nonsense mediated mRNA decay [11,12]. However, other LoF variants located within introns ±3–20 nucleotides from the splice site region that disrupt the normal pattern of mRNA splicing have been described [13,14]. It has been estimated that these splice site variants account for 5% of all pathogenic variants in *BRCA1* and *BRCA2* [15,16].

With advances in sequencing technology, there have been reports of pathogenic variants located deeper in the intronic regions (beyond ±20 nucleotides). These variants introduce new splice sites, affecting gene function [17–19]. Depending on the size and complexity of the intronic sequences, these variants can be identified by targeted whole-gene, or whole-genome or -exome sequencing (WGS or WES) methods [19]. The contribution of these variants in *BRCA1* or *BRCA2* to hereditary BC and OC is unknown due to the paucity of studies [20–22]. In vitro studies demonstrating the biological impact on splicing [15,23] have led to the reclassification of such variants of unknown clinical significance (VUS) to either pathogenic/likely pathogenic or benign/likely benign [24].

Here, we describe sequencing results for *BRCA1* and *BRCA2* derived from the application of WES and bioinformatic analyses in the re-evaluation of OC cases reported negative for pathogenic variants in these genes by clinical testing. We report the identification of an intronic variant in *BRCA1* (NM_007294.4): c.5407-25T>A harbored by sisters affected with OC. We also report the analysis of this variant in WES data from early-onset OC cases not selected for family history of cancer and OC cases from HBOC families, loss of heterozygosity (LOH) analyses of the *BRCA1* locus in OC tumor

DNA and WES analyses of peripheral blood lymphocyte (PBL) DNA of 10 other OC predisposing genes from variant carriers. We relate our observations to independent findings of all *BRCA1* intronic variants that were identified in the context of HBC and HBOC that were curated from ClinVar, a resource that aggregates information about relationships among variation and human health [8], and from a review of the literature.

2.4. Methods

5.4.1. Study Participants

All study participants were selected from biobanks available through adult hereditary cancer clinics in Quebec and/or the Banque de tissus et données of the Réseau de recherche sur le cancer of the Fond de recherche du Québec–Santé (RRCancer biobank) (rrcancer.ca), who had been recruited for research to biobanks in accordance with ethical guidelines of their respective Institutional Research Ethics Boards. All cases and information about their cancer family history, histopathology, tumor grade, disease stage and/or age of diagnoses was anonymized prior to being provided. This project was conducted with approval and in accordance with the guidelines of The McGill University Health Centre Research Ethics Board (MP-37-2019-4783).

The 27 OC cases investigated for the re-evaluation of *BRCA1* and *BRCA2* included cancers of the ovary or fallopian tubes, or primary peritoneal carcinomas. They are part of 22 OC families defined by having at least two OC cases within first-, second- or third- degree relatives and were selected from the biobanks for this study as they had been previously tested and found negative for pathogenic variants in these genes in medical genetics settings. The characteristics of 16 OC cases from 14 families of French Canadian (FC) ancestry of Quebec have been described previously [25–27]; and the remaining 11 OC cases from eight families self-reported having European ancestries. The average age at diagnosis for 25 of 27 OC cases is 55 (median: 57 and range: 25–74) years, as this data was not provided for two cases. These cases comprised serous, high-grade serous carcinoma (HGSC), high-grade endometrioid carcinoma and carcinomas of mixed OC subtypes.

The study participants investigated for the identified *BRCA1* variant have been described previously [25–27]: 53 sporadic early-onset OC cases, not selected for family

history of cancer who were diagnosed before the age of 50 years and tested negative for pathogenic *BRCA1* or *BRCA2* pathogenic variants; and 24 OC cases from 22 HBOC syndrome families who previously tested positive for pathogenic *BRCA1* or *BRCA2* variants. All cases self-reported FC ancestry of Quebec and had undergone medical genetic testing in adult hereditary cancer clinics in Quebec, as described elsewhere [27].

5.4.2. WES Analysis of BRCA1 and BRCA2 Loci

BRCA1 (NM_007294.4) and *BRCA2* (NM_000059.4) loci were investigated for pathogenic variants in PBL DNA from 27 OC cases from 22 families. These cases were subjected to WES and a customized bioinformatics pipeline for germline variant calling at the McGill Genome Center, as previously described [27]. NimbleGen SeqCap® EZ Exome Kit v3.0 (Roche, USA) was used to capture 64 mega base pairs of coding, non-coding and flanking intronic regions of up to 100 base pairs, where the average coverage of the coding region was 100x, and that of the flanking intronic region was 60x [28,29]. Then, the annotated variant call format (VCF) files were subjected to additional filtering and prioritizing criteria as follows. We filtered WES data for all rare variants in *BRCA1* and *BRCA2* with a minor allele frequency (MAF) ≤0.005 in the non-cancer general population using Genome Aggregation Database (gnomAD) v2.1.1 (gnomad.broadinstitute.org) [30,31]. This database reports on WES and WGS data from different populations, including MAF of rare pathogenic variants found in less than 1 in 10,000 individuals in the general population [9]. Loci with total coverage of <10 and/or alternative variant frequency of <0.2 or >0.8 were filtered out in order to reduce the rate of false positive variants [32]. Candidate variants were verified manually using the Integrative Genomics Viewer (IGV) v2.8 [33].

The identified candidate variant was verified in the carrier's PBL DNA using bidirectional Sanger sequencing of amplified PCR products using customized primers (forward: 5-ACAGTAGGACCTCATGTCTACA-3; and reverse: 5-ATGGAAGCCATTGTCCTCTG-3) at the McGill Genome Center, as previously described [27]. Sequencing chromatograms were then visually inspected for the variant using 4Peaks v1.8 (nucleobytes.com/4peaks/) (The Netherlands Cancer institute,

Amsterdam, The Netherlands).

5.4.3. Databases and In Silico Tools for the Evaluation of BRCA1 and BRCA2 Variants

Variants identified in *BRCA1* and *BRCA2* were assessed for their conservation and deleteriousness at RNA and protein levels using different in silico tools, which were selected based on their best predictive performance as previously described [19,34–36]. Variants were also evaluated for their clinical classification as pathogenic or likely pathogenic in the context of cancer using the ClinVar database [8], ACMG guidelines [9] and BRCA exchange database [7].

5.4.4. Surveying Carrier Frequencies in Other In-House Sequencing Data of OC Cases

We surveyed our available in-house WES data that had previously been generated from PBL DNA from different study groups to identify additional carriers of the identified *BRCA1* variant and verify *BRCA1* and *BRCA2* status. This group consisted of 53 sporadic early-onset OC cases who were reported to have tested negative in clinical settings for pathogenic variants in *BRCA1* and *BRCA2* and 24 familial OC cases from 22 HBOC syndrome families who had tested positive for pathogenic variants in *BRCA1* and *BRCA2*. All cases were subjected to the same WES capture kits and bioinformatics pipeline for germline variant calling applied for the 27 cases, as described above.

5.4.5. Profiling Tumor DNA from BRCA1 c.5407-25T>A Variant Carriers

LOH analysis was performed by Sanger sequencing of OC tumor DNA from *BRCA1* c.5407-25T>A variant carriers using customized primers as described above. Extracted DNA from fresh-frozen (FF) or histopathological sections from formalin-fixed paraffin-embedded (FFPE) tumor tissues were provided (RRCancer biobank) for DNA extraction based on the manufacturer's instructions (Promega, Canada). Sequencing chromatograms were then visually inspected for loss of the wild-type allele, as above.

5.4.6. Characterization of BRCA1 c.5407-25T>A Carriers for Co-Occurring Pathogenic Variants in Other Known OC Risk Genes

We extracted all variants in the following 10 known OC risk genes [37] from WES data from the three *BRCA1* c.5407-25T>A variant carriers and all of the remaining 25 familial OC cases: *MLH1* (NM_000249.4), *MSH2* (NM_000251.3), *MSH6* (NM_000179.3), *PMS2* (NM_000535.7), *BRIP1* (NM_032043.3), *RAD51C* (NM_058216.3), *RAD51D* (NM_001142571.2), *STK11* (NM_000455.5), *PALB2* (NM_024675.4) and *ATM* (NM_000051.4) selected based on the National Comprehensive Cancer Network (NCCN) Clinical Practice in Oncology Guidelines 2020 (Version 2.2021)-Genetic/Familial High-Risk Assessment: Breast, Ovarian and Pancreatic [38].

WES data were filtered for rare variants with MAF ≤0.005 based on the non-cancer general population using gnomAD v2.1.1 (gnomad.broadinstitute.org) [30,31]. The missense and intronic variants on this list were further prioritized as predicted to be damaging or affect splicing using in silico tools selected based on their best predictive performance [19,34–36]. Rare variants from this list were then prioritized for further review based on their clinical classification as pathogenic or likely pathogenic in the context of cancer using the ClinVar database [8] and ACMG guidelines [9].

2.5. Results

5.5.1. WES and Bioinformatics Analysis Identified BRCA1 c.5407-25T>A as a Candidate Pathogenic Variant

By applying our bioinformatics pipeline and filtering criteria on WES data from 27 OC cases from 22 families, we identified a total of four *BRCA1* and six *BRCA2* variants (**Table S2.1.**). The variants were identified in six cases from five families, meaning some OC cases harbored more than one variant, and as described further below, there was one family of siblings harboring an identical *BRCA1* variant.

From the list of seven exonic variants identified by our methods, all but *BRCA2* c.4570T>G; p.Phe1524Val were not predicted to be damaging by all seven selected in silico tools (see **Table S2.1.**). Furthermore, all these coding variants have been

classified as benign by ClinVar and/or benign or likely benign by ACMG guidelines in the context of hereditary BC and/or OC and reported as benign in the BRCA exchange database [7]. These observations are consistent with medical genetic reports for cases harboring these variants, as commercial testing should have detected these exonic variants if regions were adequately covered.

The three intronic *BRCA1* variants identified by our methods are interesting as they may not have been detectable by commercial testing. The allele frequencies of BRCA1 c.134+1335del and c.4358-722del are unknown, as neither variant was identified in gnomAD. In contrast, in gnomAD BRCA1 c.5407-25T>A is infrequent in the non-cancer general population having a MAF of 7.46x10−6, and a carrier frequency of two out of 134,138 individuals from the non-Finnish European population, a population of ancestral origin closest to the ancestry of our cancer families. As these intronic variants are located beyond ±20 nucleotides from splice sites, none of the prediction scores for affecting splicing were generated by the in silico tools Maximum Entropy Estimates of Splice junction strengths v2.0 (MaxEntScan v2.0) [39], Human Splicing Finder v3.1 (HSF v3.1) [40] and two Database Splicing Consensus Single Nucleotide Variant (dbscSNV) in silico tools: AdaBoost v4.0 (ADA v4.0) and Random Forest v4.0 (RF v4.0) [41] (**Table S2.1.**). Therefore, we used SpliceAI, a relatively new in silico tool based on a deep learning and pre-mRNA transcript sequencing database, which generates different scores between 0 and 1 that can be interpreted as the probability of the variant affecting splicing by the loss or gain of a splice acceptor or a splice donor site [19]. SpliceAI predicted *BRCA1* c.5407-25T>A may result in splice acceptor loss (delta score for acceptor loss = 0.41) (**Table S2.1.**), suggesting that it might exert a deleterious effect on the transcription of *BRCA1*. Moreover, the locus of *BRCA1* c.5407-25T>A is predicted to be conserved by in silico tools, supporting its biological importance [42]. In contrast, the other two intronic variants were not predicted to affect splicing of *BRCA1* by SpliceAI (**Table S2.1.**). Using IGV, a manual inspection of the sequencing reads for *BRCA1* c.134+1335del and c.4358-722del revealed that they are located within repetitive regions deep within introns 3 and 12 of *BRCA1*, respectively, suggesting that sequencing data could be due to technical artifacts [43]. Indeed, association with risk is questionable as both intronic variants have been classified as

benign in ClinVar and by ACMG guidelines, though they had not yet been reviewed in the BRCA exchange database (**Table S2.1.**).

BRCA1 c.5407-25T>A is located at base pair 25 of intron 21 upstream from the start of exon 22 based on the transcript NM_007294.4 (**Figure 1-A**), or of intron 22 upstream from the start of exon 23 based on the canonical transcript NM_007300.4, a transcript containing exon 4, which was missed due to a historical mis-annotation of an additional exon 4 in *BRCA1* [44]. In this report, we have annotated our variants using the *BRCA1* transcript (NM_007294.4), as it is the commonly used in the clinical genetic setting. Interestingly, this variant was identified in two affected sisters with cancer (**Figure 2.1-B**), and a manual review of their sequencing files using IGV (**Figure 2.1-A**) shows an average coverage of 60 by our WES capture kit as has been demonstrated by gnomAD v2.1 WES data (gnomad. broadinstitute.org/gene/ENSG00000012048?dataset=gnomad_r2_1 accessed on 5 February 2021). The variant was verified by bidirectional Sanger sequencing of PBL DNA (**Figure 2.1-C**) from both of our carriers.

5.5.2. WES Analyses Identified Another OC Case Harboring BRCA1 c.5407-25T>A

To determine whether *BRCA1* c.5407-25T>A occurs in other OC cases from our study groups, we reviewed similarly derived WES data sets from familial and sporadic OC cases. This variant was not identified in WES data from 24 OC cases from HBOC families harboring pathogenic *BRCA1* or *BRCA2* variants. In contrast, a carrier was identified among 53 OC cases who developed HGSC before the age of 50 years. Interestingly, this case (PT0198) had previously been reported as negative for pathogenic variants in *BRCA1* or *BRCA2* as well as in *MLH1*, *MSH2*, *MSH6* and *PMS2*. The sporadic cancer case harboring the *BRCA1* variant was diagnosed with HGSC at the age of 45 years, and the variant was verified by bidirectional Sanger sequencing of their PBL DNA (**Figure 2.1-D**).

5.5.3. WES Analyses of BRCA1 c.5407-25T>A Carriers Suggest That They Are Unlikely to Harbor Pathogenic Variants in the Other Known OC Risk Genes

To further support the role of *BRCA1* c.5407-25T>A in OC risk, we extended our WES

and bioinformatic analyses to include an investigation of other known OC risk genes

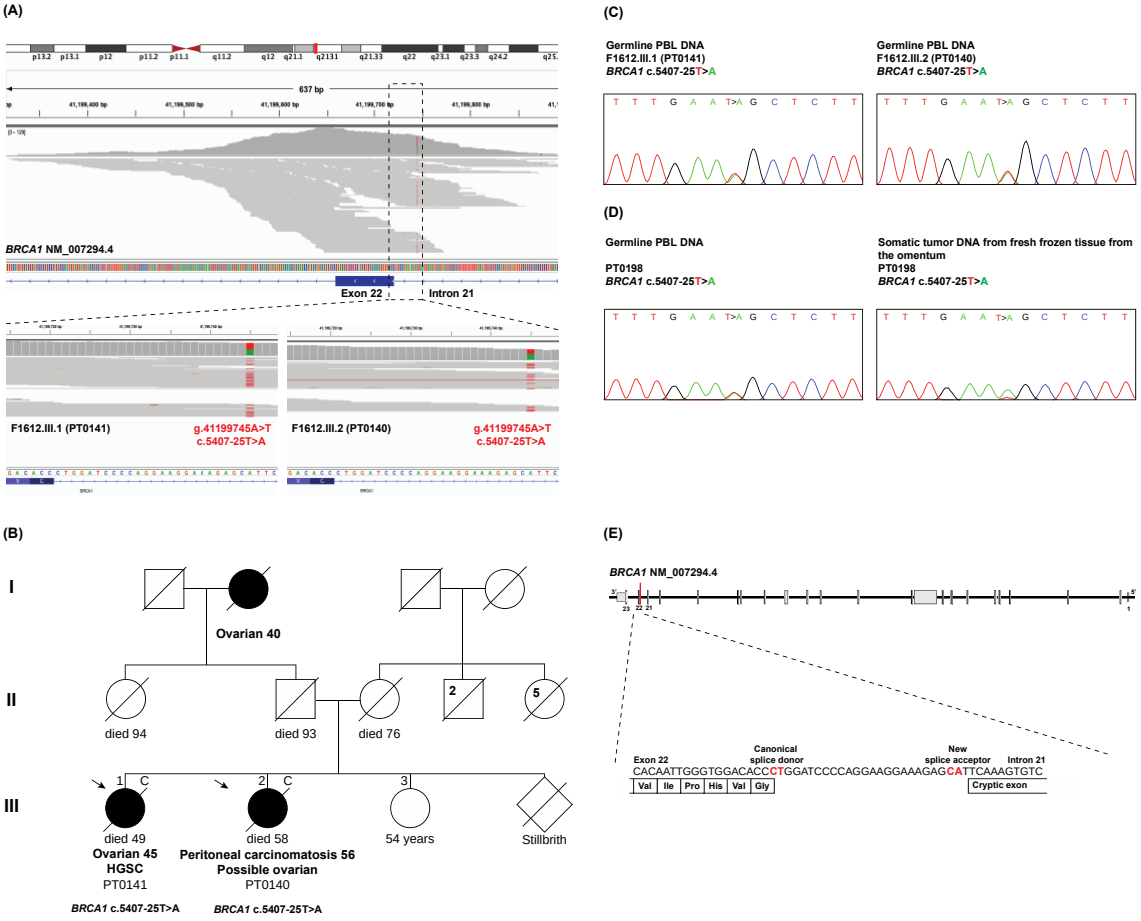

Figure 2.1. Identification of *BRCA1* c.5407-25T>A, a likely pathogenic intronic variant.

(A) Integrative Genomics Viewer (IGV) v2.8 sequencing data showing coverage of exon 22 of BRCA1 and flanking intronic regions beyond ±20 nucleotides of location of BRCA1 c.5407-25T>A in variant carriers; (B) pedigree of BRCA1 c.5407-25T>A carrier family (F1612) indicating confirmed (“C”) cases of bilateral high-grade serous carcinoma (HGSC) (PT0141) and primary peritoneal carcinomatosis with possible ovarian origin (PT0140) by pathology reports; (C) sequencing chromatogram of peripheral blood lymphocytes (PBL) DNA verifying heterozygous carrier status of both sisters; and (D) sequencing chromatograms from a sporadic early-onset OC case (PT0198) verifying heterozygous BRCA1 variant carrier status in PBL DNA and loss of the wild-type allele

in OC tumor DNA; (E) Schema illustrating the location of the intronic BRCA1 variant creating a new splice site as predicted by SpliceAI.

[38] in the familial OC (PT0141 and PT0140, see **Figure 2.1-B**), and early onset OC (PT0198) cases harboring this *BRCA1* allele. A review of WES data for pathogenic variants in *MLH1*, *MSH2*, *MSH6*, *PMS2*, *BRIP1*, *RAD51C*, *RAD51D*, *PALB2*, *ATM* or *STK11* identified a carrier of *MSH6* (NM_000179.2): c.-18G>T in PT0140 from family F1612. This variant is classified as benign or likely benign by six submissions in ClinVar (Accession number VCV000089159.7) and likely benign by ACMG guidelines. A similar analysis of the WES data from PT0198 did not identify any pathogenic variants in these genes.

A similar analysis of WES data in remaining 25 familial OC cases did not identify any variants classified as pathogenic or likely pathogenic in any of the 10 OC risk genes. Indeed the only rare variant identified, a missense variant in *BRIP1* (NM_032043.3): c.2220G>T; p.Gln740His harbored in two sisters (PT0204 and PT0217) from family F1608 was not predicted to be damaging by five out of the seven selected in silico tools. Furthermore, this variant has been classified as likely benign by 6 submissions and VUS by 15 submissions in ClinVar (VCV000133752.33) and VUS by ACMG guidelines in the context of hereditary BC and/or OC.

However, our analyses of the sporadic early-onset OC case (PT0198), which included a thorough investigation of *BRCA1* and *BRCA2* loci, revealed that they also harbored *BRCA2* c.1938C>T; p.Ser646Ser, a variant also found in one of the familial OC carrier cases. As discussed above, this synonymous variant in *BRCA2* is classified as benign (**Table S2.1.**). Thus, our findings suggest that *BRCA1* c.5407-25T>A carriers are unlikely to harbor pathogenic, likely pathogenic or VUS in the other known OC predisposing genes based on NCCN guidelines for clinical practice in oncology [38].

5.5.4. LOH Analysis of the Tumor DNA from BRCA1 c.5407-25T>A Carrier Revealed Loss of the Wild-Type Allele

We performed LOH analysis of tumor DNA from PT0198, the only available OC tumor DNA from *BRCA1* c.5407-25T>A carriers. DNA sequencing analyses suggested loss of the wild-type allele had occurred in the development of OC in this case (**Figure 2.1-D**).

This finding is consistent with *BRCA1* c.5407-25T>A, playing a role in OC risk, as has been shown with LOH analyses of OC tumor DNA from carriers of *BRCA1* pathogenic variants [45].

2.6. Discussion

Our WES and bioinformatics analyses of 27 familial OC cases who had undergone medical genetic testing and who were reported as negative for pathogenic variants in *BRCA1* and *BRCA2* identified two sisters harboring *BRCA1* c.5407-25T>A. Further investigation of WES data from additional OC cases identified another carrier of this rare *BRCA1* variant among the cases who were also previously reported as having tested negative for pathogenic variants in *BRCA1* or *BRCA2*. As our WES data captured some intronic sequencing data, it is possible that this variant was not detected by medical genetic testing efforts due to commercial testing protocols that were applied.

Our application of SpliceAI, a new tool capable of predicting splice sites up to 10 kilobase pairs from the exon-intron junctions [19], predicted that this intronic variant may result in a splice acceptor loss. This suggests that the nucleotide substitution at -25 from exon 22 along with the adjacent nucleotide at -24 created a new splice acceptor site, potentially resulting in the loss of the entire or part of exon 22 of *BRCA1* (see **Figure 2.1-E**). The sensitivity of predicting aberrant splicing effects is estimated to be at least 70% for intronic variants between ±20–50 base pairs from exon–intron junctions [19]. Intronic regions containing sequences of potential exonic characteristics are referred to as pseudoexons or exons where a single substitution or small deletion or insertion may create new splice sites, such that these pseudoexons would be recognized by splicing machinery and result in abnormal patterns of splicing [17,46]. The application of SpliceAI has been used recently in different disorders [47].

BRCA1 c.5407-25T>A was identified in siblings both having OC (one diagnosed with a HGSC of the ovary and the other with primary peritoneal carcinomatosis), cancer phenotypes consistent with harboring a *BRCA1* or *BRCA2* pathogenic variant [48,49]. Indeed, applying the Manchester Scoring System revealed a probability greater than 10% of either sibling harboring a pathogenic variant in *BRCA1* (score = 23) or *BRCA2*

(score = 15) [50–52]. Interestingly, we also found this variant in one of the early-onset OC cases who developed HGSC before the age of 50 years, which is consistent with observations that the average age of diagnosis of HGSC is less than 60 years of age in carriers of pathogenic *BRCA1* variants [3]. Moreover, our genetic analysis of OC tumor DNA from this carrier revealed the loss of the wild-type allele and retention of the *BRCA1* variant allele. This observation is consistent with *BRCA1* c.5407-25T>A, playing a role in OC risk, as has been shown with LOH analyses of OC tumor DNA from those harboring LoF pathogenic variants in *BRCA1* [45]. *BRCA1* c.5407-25T>A had initially been classified as a VUS in ClinVar based on two submissions of its identification in the context of HBC or HBOC (**Table S2.1.**) and five independent studies published prior to 2020 that also described its identification in this hereditary cancer context (**Table 2.1.**). The *BRCA1* variant was identified via different detection platforms such as protein truncation test, single-strand conformational polymorphism analysis [53] or multiplex ligation-dependent probe analysis. In 2020, during the course of our investigation, Høberg-Vetti et al. reported the identification of *BRCA1* c.5407-25T>A in BC (n = 12) and OC (n = 8) cases, which also included a case of peritoneal carcinomatosis, in 20 cancer families with *BRCA1* Manchester scores ranging from 3 to 30 [54]. Indeed, the authors mentioned that they had identified this variant as early as 2006 and had evidence from one case of an effect on *BRCA1* transcript [55], though the results were not published [54], highlighting the complexity of interpreting intronic variants. The prevalence of this variant reported in the Norwegian study groups (see **Table 2.1.**) suggests the possibility of common ancestry for carriers, as has been shown with specific pathogenic variants in defined populations from our study of French Canadians and described in other studies [36]. Høberg-Vetti et al. also provided evidence that *BRCA1* c.5407-25T>A creates a new splice site, resulting in the skipping of exon 22, based on a deletion of 61 nucleotides deduced from sequencing the corresponding aberrant size transcript [54]. This could affect protein function as it would result in the partial deletion of the BRCA1 Carboxy-Terminus (BRCT) domain (**Figure 2.2.**), and thereby affect the binding of several proteins such as BRIP1, RAP80 and CtIP, which mediate the recruitment or stability of BRCA1 [56]. However, this group also demonstrated that the shift in the reading frame, which introduces a premature

termination codon after 11 amino acids BRCA1 p.Gly1803GlnfsTer11, likely triggers nonsense-mediated mRNA decay [54]. As RNA is not available from our *BRCA1* c.5407-25T>A carriers, we are unable to replicate these findings. Although more research on OC risk associated with *BRCA1* c.5407-25T>A is required, collectively, these observations are supportive of the ClinVar classification of likely pathogenic rather than VUS.

Table 2.1. Features of *BRCA1* c.5407-25T>A carriers from independent reports.

Year Reported	Population[1]	Number of Carriers per Study Group	Cancer Type in Carriers	Study Group Investigated[2]	Reference
2003	Germany	1/90	Breast	Early-onset cases not selected for family history of cancer	[53]
2014	Greece	1/473	Breast	HBC and HBOC families	[57]
2016	Norway	2/893	Breast	Cases not selected for family history of cancer	[55]
2018	Norway	9/669	Breast	HBC and HBOC families	[58]
2019	Norway	8/1914	Breast or ovarian	Sporadic cases and families	[59]
2020	Norway, France, United States of America	20	Breast or ovarian	Selected HBC and HBOC families	[54]
2022	French Canadian, Ashkenazi Jewish, Austria, United Kingdom, Germany, Italy	2/27	Ovarian	Families with at least two OC case within first-, second- or third-degree relatives	This report
2022	French Canadian	1/53	Ovarian	Sporadic OC case with early onset of the disease not selected for family history of cancer	This report

[1] Geographic origin of population or self-reported as French Canadian from Quebec.

[2] Cases investigated include hereditary breast cancer (HBC) and hereditary breast and ovarian cancer (HBOC) families.

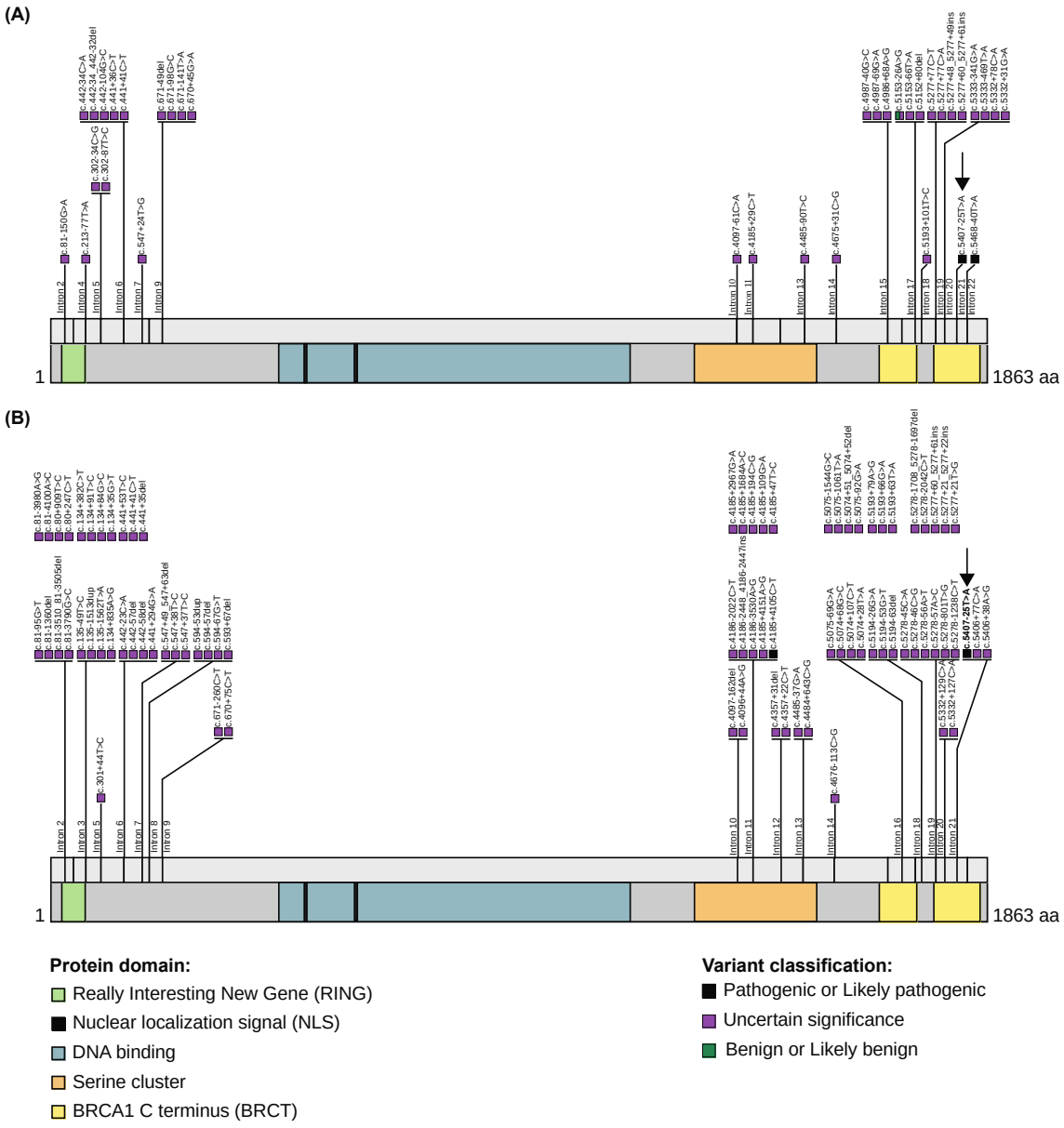

Figure 2.2. Annotated curated intronic *BRCA1* variants.

The *BRCA1* transcript NM_007294.4 (NCBI Reference Sequence (RefSeq) database (ncbi.nlm.nih.gov/nuccore/NM_007294.4), indicating protein encoded domains was annotated with BRCA1 c.5407-25T>A (indicated with an arrow) and intronic variants classified as pathogenic, likely pathogenic or unknown significance based on ClinVar and/or American College of Medical Genetics and Genomics guidelines were (A) submitted to ClinVar (ncbi.nlm.nih.gov/clinvar/ accessed on 25 January 2022) (see **Table S2.2.**) or (B) reported in the literature (pubmed.ncbi.nlm.nih.gov accessed on 25 January 2022) (see **Table S2.3.**). Variants were selected based on: intronic location

beyond ±20 nucleotides from splice sites; rarity (minor allele frequency ≤0.005); classification as pathogenic, likely pathogenic or variants of unknown significance (VUS) in BRCA1 in the context of hereditary breast and/or ovarian cancer Search terms for PubMed (as in October 2021) articles included: ("brca1 s"[All Fields] OR "genes, brca1"[MeSH Terms] OR ("genes"[All Fields] AND "brca1"[All Fields]) OR "brca1 genes"[All Fields] OR "brca1"[All Fields]) AND ("introns"[All Fields] OR "intron"[All Fields] OR "intronically"[All Fields] OR "intronization"[All Fields] OR "introns"[MeSH Terms] OR "introns"[All Fields] OR "intron"[All Fields] OR "intronic"[All Fields]) AND ("variant"[All Fields] OR "variant s"[All Fields] OR "variants"[All Fields]). Intergenic, 3'UTR and 5'UTR variants and large chromosomal rearrangements were excluded.

The frequency of pathogenic *BRCA1* intronic variants is currently unknown but likely underreported due to complex methods used to identify them and assess their biological and clinical impact. While researching independent evidence for the pathogenicity of *BRCA1* c.5407-25T>A, we surveyed the ClinVar database (**Figure 2.2-A** and **Table S2.2.**) for rare, intronic *BRCA1* variants located beyond ±20 nucleotides, rationalizing that this resource would report variants with biologically meaningful associations with cancer risk. A literature review revealed that intronic variants are being identified using a variety of DNA and RNA sequencing technologies, including reverse-transcribed- [60,61], long-range- [62] and multiplex- [63] PCR-based assays, some of which aim to identify variants within intronic regions as large as 10 kilobase pairs [15]. Recently, next-generation sequencing technologies involving RNA [64] or whole genome [18,65] sequencing have been applied. The biological impact of intronic variants can be difficult to discern but usually involve in cellulo assays of genetically engineered cell lines sometimes derived from carriers or minigene constructs [66,67]. Our survey of the ClinVar database revealed that 0.3% (35/11,366) of all reported BRCA1 variants were rare intronic variants that met our criteria (see **Figure 2.2-A**), where 46% (16/35) were identified between ±20 and ±50 nucleotides and the remaining beyond ±50 nucleotides from exonic-intronic junctions. In total, 2 of the 35 intronic variants were listed as having conflicting interpretation, the variant of our interest c.5407-25T>A (VCV000371817) as VUS or likely pathogenic and c.5153-26A>G (VCV000125786) as VUS or likely benign;

and the remaining 33 variants were classified as VUS. Only 3 of 35 BRCA1 intronic variants listed in ClinVar were identified in the gnomAD database, and this included our variant of interest, BRCA1 c.5407-25T>A (**Table S2.2**.).

From a literature review, we curated a list of 223 original research studies or case reports about rare intronic variants in *BRCA1* (**Figure 2.2-B** and **Table S2.3.**). This list included 32 reports describing 80 such variants. Of these intronic variants, 21% (17/80) were identified between ±20 and ±50 nucleotides and the remaining beyond ±50 nucleotides from exonic–intronic junctions. Only 2 of 80 variants had been classified as pathogenic c.4185+4105C>T (VCV000632611.2) or likely pathogenic c.5407-25T>A, our variant of interest, and the remaining as VUS. Only 4 of the 80 variants were identified in the ClinVar database, and all had been classified as VUS based on ACMG guidelines [9].

Some studies have argued that the majority of deep intronic variants are unlikely to be associated with cancer risk [68]. Interestingly, the frequency of intronic variants predicted by in silico tools to affect splicing is comparable to those predicted to effect bona fide splice site regions [19,69]. We applied SpliceAI [19] to predict the effect in splicing of *BRCA1* to the *BRCA1* intronic variants identified in ClinVar and in our literature search. Unlike our findings with *BRCA1* c.5407-25T>A, SpliceAI predicted that the majority of curated intronic variants would not affect splicing, though the accuracy of this in silico tool did not reach 95% for all applications (see **Table S2.3.**). New in silico tools have been developed to predict the splicing impact by these intronic variants using different mathematical models such as CADD-Splice [69] and SQUIRLS [70]. None of these tools have been tested yet on hereditary cancer syndromes. Earlier developed in silico predictive models include MaxEntScan [39], HSF [40] or both dbscSNV tools [41]: ADA or RF were designed to predict variants within the splice regions. These tools have been tested on different datasets including sequencing data from hereditary BC and OC cases [71]. However, these in silico tools are limited to predicting events that occur within splice regions. Although in cellulo assays would provide supportive evidence for biological impact predicted by bioinformatic tools, the causality of an intronic variant identified in an established highly penetrant CPG such as *BRCA1* in conferring risk to cancer remains a challenge.

Clinical testing for pathogenic variants in *BRCA1* and *BRCA2* has been established in clinical settings as it has been proven to improve cancer risk assessment and management of carriers [37]. OC cases harboring pathogenic variants in these genes are also offered targeted chemotherapies based on poly (ADP-ribose) polymerase (PARP) inhibitors as part of the standard of care treatment regimens, as carriers have shown improvement in overall outcome [72,73]. As sequencing information is gathered from OC patients undergoing different treatment regimens, it will be interesting to investigate the response to PARP inhibitors in carriers of deep intronic variants, particularly those predicted to affect splicing and impact gene function by in silico analyses or by in cellulo assays. Our survey of ClinVar and the literature identified 105 rare intronic *BRCA1* variants that are classified as VUS for further confirmation of their pathogenicity. Although our study may have been limited by WES, our report highlights the importance of the comprehensive sequencing of the entirety of *BRCA1* and *BRCA2* to capture all possible pathogenic variants in individuals at risk for hereditary OC and BC.

2.7. Conclusion

Using our WES and bioinformatics analyses, we were able to identify an intronic variant in *BRCA1* in one OC family who had tested negative for pathogenic variants in *BRCA1* or *BRCA2* by commercial testing. We also identified this variant in another OC case diagnosed at an early age and showed loss of the wild-type allele in the tumor DNA using LOH analysis. A splice predictor algorithm suggests that it exerts aberrant splicing affecting gene function. Our findings support *BRCA1* c.5407-25T>A as a likely pathogenic variant and highlights the importance of investigating any intronic variants as causal variants in OC families where the involvement of *BRCA1* is highly suggestive.

2.8. Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13040697/s1 (also see **Appendix II**).

2.9. Acknowledgment

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2.10. Authors contributions

Conceptualization, W.M.A. and P.N.T.; methodology, W.M.A., T.R., C.T.F., J.R. and P.N.T.; investigation and formal analyses, W.M.A., C.T.F., C.S., J.R. and P.N.T.; cases resources: A.-M.M.-M., D.P., W.D.F. and Z.E.H.; writing—original draft preparation, W.M.A. and P.N.T.; writing—reviewing and editing, T.R., C.T.F., C.S., A.-M.M.-M., D.P., Z.E.H., J.R. and P.N.T.; and supervision, P.N.T. All authors have read and agreed to the published version of the manuscript.

2.11. Conflict of interest

The authors declare no conflict of interest.

2.12. Ethical approval

This project was conducted and approved according to the guidelines of The McGill University Health Centre Research Ethic Board (MP-37-2019-4783) (see **Appendix IX**).

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CHAPTER III: The Genetic and Molecular Analyses of *RAD51C* and *RAD51D* Identifies Rare Variants Implicated in Hereditary Ovarian Cancer from a Genetically Unique Population

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3.1. Preface

At the time of the discovery of *BRCA1* and *BRCA2* as ovarian cancer predisposing genes, it was known that not all ovarian cancer families could be explained by pathogenic variants in either of these genes. This implied that other ovarian cancer risk genes had yet to be discovered. Fifteen years later, *RAD51C* and *RAD51D* were the first additional ovarian cancer predisposing genes to be discovered. The French Canadian population of Quebec has been recognized for its contribution to research in medical genetics in characterizing new ovarian cancer risk variants in previously reported ovarian cancer predisposing genes due to its unique genetic architecture. Dr. Patricia Tonin and her group were the first to report: 1) specific pathogenic variants in the established ovarian cancer risk genes, *BRCA1* and *BRCA2,* that are frequently occurring in ovarian cancer families and cases in the French Canadian population; and 2) that the ovarian cancer carriers of such variants shared the same haplotype suggesting these chromosomal segments containing each variant were inherited from a common ancestor. After the discovery of *RAD51C* or *RAD51D* and at the time of starting my Ph.D. program, there were two reports that no risk variants were identified in either of these genes in the ovarian cancer families and cases from the French Canadian population of Quebec.

In this chapter, I applied a series of strategies which are presented in this thesis to: (1) identify new candidate risk ovarian cancer variants in *RAD51C* or *RAD51D* using whole exome sequencing and bioinformatics analyses of the germline of ovarian cancer families and cases of French Canadian ancestry of Quebec, who I confirmed were negative for pathogenic *BRCA1* and *BRCA2* variants; and (2) determine the prevalence of these candidate risk variants in ovarian cancer cases and controls of French Canadian ancestry.

3.2. Abstract

To identify candidate variants in *RAD51C* and *RAD51D* ovarian cancer (OC) predisposing genes by investigating French Canadians (FC) exhibiting unique genetic architecture. Candidates were identified by whole exome sequencing analysis of 17 OC families and 53 early-onset OC cases. Carrier frequencies were determined by the genetic analysis of 100 OC or HBOC families, 438 sporadic OC cases and 1025 controls. Variants of unknown function were assayed for their biological impact and/or cellular sensitivity to olaparib. *RAD51C* c.414G>C;p.Leu138Phe and c.705G>T;p.Lys235Asn and *RAD51D* c.137C>G;p.Ser46Cys, c.620C>T;p.Ser207Leu and c.694C>T;p.Arg232Ter were identified in 17.6% of families and 11.3% of early-onset cases. The highest carrier frequency was observed in OC families (1/44, 2.3%) and sporadic cases (15/438, 3.4%) harbouring *RAD51D* c.620C>T versus controls (1/1025, 0.1%). Carriers of c.620C>T (n = 7), c.705G>T (n = 2) and c.137C>G (n = 1) were identified in another 538 FC OC cases. *RAD51C* c.705G>T affected splicing by skipping exon four, while RAD51D p.Ser46Cys affected protein stability and conferred olaparib sensitivity. Genetic and functional assays implicate *RAD51C* c.705G>T and *RAD51D* c.137C>G as likely pathogenic variants in OC. The high carrier frequency of *RAD51D* c.620C>T in FC OC cases validates previous findings. Our findings further support the role of *RAD51C* and *RAD51D* in hereditary OC.

Keywords: *RAD51C*; *RAD51D*; ovarian cancer predisposing genes; French Canadian; genetic drift.

3.3. Introduction

Ovarian cancer (OC) has a high estimated heritable component (39%, 95% confidence interval [CI]: 23–55) [1]. Harbouring loss-of-function (LoF) variants in *BRCA1* [2] or *BRCA2* [3] confers significant lifetime risk of developing OC, which accounts for 40–85% of OC cases in hereditary breast and ovarian cancer (HBOC) syndrome families and 10–15% of those not selected for age at OC diagnosis and/or family history of cancer [4]. Carrying rare LoF variants in *RAD51C* [5] and *RAD51D* [6] has been associated with OC predisposition in different populations [7], though the penetrance has yet to be determined. These genes encode RAD51 paralog proteins that are structurally similar to

the RAD51 recombinase, which allows faithful DNA double-strand break repair along with BRCA1 and BRCA2 by the homologous recombination (HR) pathway [8]. RAD51C [9] and RAD51D [6] deficient cells or those expressing pathogenic variants in these genes [6,10] have been shown to render sensitivity to poly (ADP-ribose) polymerase (PARP) inhibitors such as olaparib, which is the first to be approved for OC treatment [11]. Indeed, four PARP inhibitors are currently approved for clinical use: olaparib, rucaparib, niraparib, and talazoparib for the treatment of *BRCA1* and *BRCA2* pathogenic variant-positive OC, breast, pancreatic and prostate cancers (recently reviewed [11]). PARP inhibitors, including olaparib, have been proven effective in the treatment of OC in the context of harbouring pathogenic *BRCA1* and *BRCA2* variants [12–16]. Thus, identifying carriers of pathogenic variants in *RAD51C* and *RAD51D* might be useful for identifying those who might benefit from management of OC with protocols using novel PARP inhibitors. Less than 2% of OC cases have been reported to harbour LoF pathogenic variants in *RAD51C* or *RAD51D* [7,17–19]. However, the role of pathogenic rare missense variants in these genes has not been fully explored, although approximately 39% are predicted to be damaging by in silico tools [17].

Investigating populations exhibiting unique genetic architecture due to common ancestors has the potential to facilitate the characterization of pathogenic variants in known or candidate cancer predisposing genes [20–22]. Our research on the French Canadians (FC) of Quebec (Canada), has shown that a small number of specific pathogenic variants account for the majority of *BRCA1* or *BRCA2* implicated HBOC and hereditary breast cancer (HBC) syndrome families, whereas a vast spectrum of variants in these genes has been reported in the general population [21,23,24]. Likewise, only one pathogenic variant accounts for all *PALB2* implicated FC HBC syndrome families [21,25,26]. The unique genetic architecture of FCs has been attributed to genetic drift as result of multiple waves of localized population expansion in Quebec of European (France) settlers since 1608 [20,21,27]. Given the expectation that a higher frequency of carriers of rare variants would be observed in cancer cases versus controls in populations exhibiting genetic drift, the genetic analyses of FCs have the potential to identify clinically relevant pathogenic variants in new risk genes [20–22]. For example, recently, we reported that *RAD51D* c.620C>T; p.Ser207Leu, initially identified by genetic

panel testing of familial OC cases in clinical settings, was found to occur at a significantly higher frequency in FC OC cases versus controls [28]. At the time of discovery, this *RAD51D* variant was classified as a missense variant of unknown clinical significance (VUS). Its classification has since been revised to pathogenic or likely pathogenic, especially as the same study reported that *RAD51D* p.Ser207Leu impaired HR function and rendered cells sensitive to olaparib. Although the role of *RAD51C* and *RAD51D* have yet to be fully explored in the FC population [21,29,30], it is clear from previous work that investigating the FC population can assist in characterizing new cancer risk genes (reviewed in [21]).

The main aim of this study was to identify and investigate candidate variants in *RAD51C* and *RAD51D* in FCs with OC by: (1) performing whole exome sequencing (WES) and bioinformatic analyses of the germline of familial and sporadic early-onset OC cases; (2) determining the carrier (harbouring the variant in the heterozygous state) frequencies of candidate variants by genotyping or surveying available genetic data in OC cases and population-matched controls; (3) assaying available tumour DNA from carriers for loss of heterozygosity (LOH) of *RAD51C* and *RAD51D* loci; (4) describing associated clinico-pathological features of OC in carriers; and (5) using biological assays involving cancer cell line models to assess the impact of missense variants with unknown biological function, including sensitivity to PARP inhibitors.

3.4. Methods

3.4.1. FC Study Participants

The FC participants investigated in this study were selected from independently established biobank resources as described in **Table S3.1.** All participants were recruited independently to these biobanks in accordance with ethical guidelines of their respective Institutions Research Ethics Boards, including those participants from adult hereditary cancer clinics in Quebec. The participants that had provided their associated biological specimens, genetic data, pedigree information and clinical metrics, where appropriate, were anonymized at source by the respective biobanks. To further protect the anonymity of study subjects, all samples were assigned a unique identifier and pedigrees modified. This project was conducted with approval and in accordance with

the guidelines of The McGill University Health Centre Research Ethics Board (MP-37-2019-4783).

As described in **Figure 3.1-A**, candidate variants in *RAD51C* and *RAD51D* were discovered (phase I) in peripheral blood lymphocyte (PBL) DNA from 20 familial (from 17 OC families) and 53 sporadic early-onset OC cases known to be negative for pathogenic *BRCA1* and *BRCA2* variants by FC mutation-panel or clinical multi-gene panel testing from information provided by adult hereditary cancer clinics in Quebec or the Banque de tissus et données of the Réseau de recherche sur le cancer of the Fond de recherche du Québec—Santé (RRCancer biobank) (rrcancer.ca).

Carrier frequencies of *RAD51C* and *RAD51D* candidate variants were determined (phase II) by genotyping PBL DNA from index cancer cases from three different FC cancer groups: 44 OC and 56 HBOC families, and 438 sporadic OC cases regardless of the carrier status of *BRCA1* and *BRCA2* pathogenic variants (RRCancer biobank). Carrier frequencies were also determined by investigating genetic data available from population-matched controls from three established biobanks: (1) CARTaGENE (cartagene.qc.ca) [31]; (2) McGill University—Montreal Neurological Institute (MNI) [28,32]; and (3) Sherbrook University—glucose regulation in gestation and growth (Gen3G) [33].

To increase the pool of carriers of our candidate variants (phase III), we genotyped another 538 PBL or tumour DNA samples from recently available OC cases that were provided by the RRCancer biobank**.**

Age at diagnosis, tumour grade, stage of the disease, histopathology subtype as confirmed by a gynecologic pathologist, personal history of other cancers, chemotherapy treatment and/or overall outcome were provided for selected OC cases from the RRCancer biobank if available. For comparative purposes, clinical data was also provided by the RRCancer biobank from carriers of the pathogenic *BRCA1* c.4327C>T; p.Arg1443Ter from the sporadic OC case study group, investigated previously by our group for *BRCA1* and *BRCA2* carrier status [34].

All study groups described in this report were selected from participants that had been recruited from the province of Quebec to participate in various biobanking projects: familial OC or invasive breast cancer (BC) cases self-reported FC ancestry [23,24]; the

majority of sporadic OC (at least 88%) self-reported FC ancestry [34]; additional OC cases self-reported FC ancestry by RRCancer biobank; Gen3G project included mothers that self-reported FC ancestry [33]; MNI controls were self-reported as FC ancestry [28,32]; and CARTaGENE resource defined FC status of controls based on being self-reported as FC, born in Quebec, having parents and all four grandparents born in Canada and having French as first language learned [31].

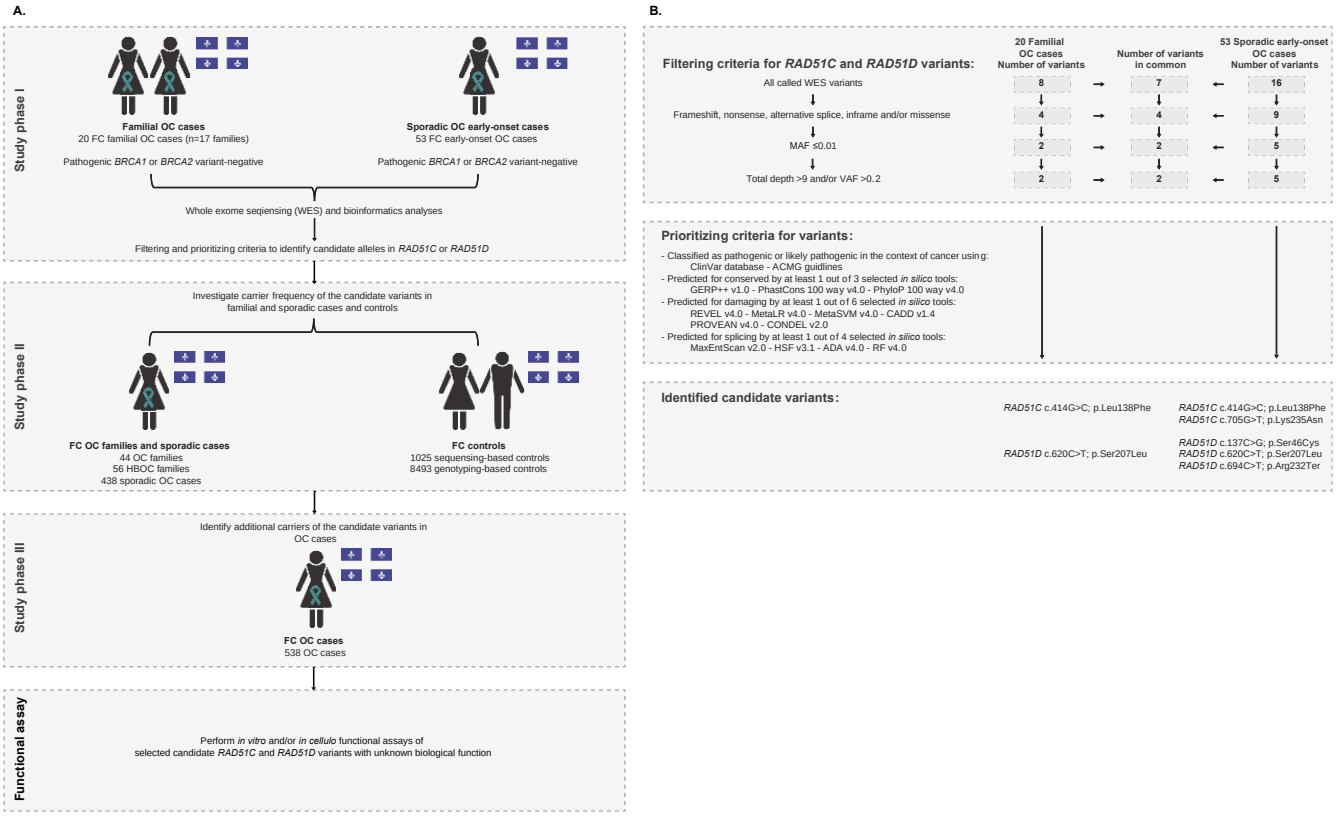

Figure 3.1. Study design and criteria for identifying candidate variants in *RAD51C* and *RAD51D*.

(A) Schematic diagram summarizing phase I of the study for identifying candidate variants in ovarian cancer (OC) cases, phase II of the study for determining the carrier frequency of the candidate variants in familial and sporadic OC cases, Hereditary Breast and Ovarian Cancer (HBOC) syndrome families and controls from the French Canadian (FC) population of Quebec, and phase III of the study for identifying additional carriers; teal ribbon signifies women with OC; and diagrams contain provincial flag of Quebec

denoting geographic ascertainment of cases and controls; and (B) Schematic diagram presenting the filtering and prioritizing criteria applied to identify candidate variants. Variants were prioritized using different in silico tools for conservation: Genomic Evolutionary Rate Profiling v1.0 (GERP++ v1.0 (score ≥ 2.0)); Phylogenetic p value v4.0 of 100 vertebrates (PhyloP 100 way v4.0 (score ≥ 0.2)) and PHAST Conservation v4.0 of 100 vertebrates (PhastCons 100 way v4.0 (score ≥ 0.4)); for predicting damaging effects based on their best predictive performance: Rare Exome Variant Ensemble Learner v4.0 (REVEL v4.0 (score ≥ 0.4)); Meta-analytic Logistic Regression v4.0 (MetaLR v4.0 (score ≥ 0.4)); Meta-analytic support Vector Machine v4.0 (MetaSVM v4.0 (score ≥ 0.0)); Combined Annotation Dependent Depletion v1.3 (CADD v1.4 (Phred score ≥ 20)); Protein Variation Effect Analyzer v4.0 (PROVEAN v4.0 (score ≥ −2.5)); and CONsensus DELeteriousness v2.0 (CONDEL v2.0 (score ≥ 0.4)); and for affecting alternative splicing: Maximum Entropy Estimates of Splice junction strengths v2.0 (MaxEntScan v2.0); Human Splicing Finder v3.1 (HSF v3.1); and two different Database Splicing Consensus Single Nucleotide Variant (dbscSNV) in silico tools: AdaBoost v4.0 (ADA v4.0 (score ≥ 0.4)) and Random Forest v4.0 (RF v4.0 (score ≥ 0.4)) (see Materials and Methods Section 2.2.).

3.4.2. Identification and Verification of Candidate Variants

To identify candidate variants, PBL DNA from a total of 73 OC phase I cases (**Figure 3.1-A**) were subjected to WES at the McGill Genome Centre using NimbleGen SeqCap EZ Exome v3.0 library kit (Roche, NJ, USA), followed by paired-end sequencing of 150 bp reads on different Illumina HiSeq platforms. Reads were aligned to the human reference genome assembly GRCh37/hg19 using Burrows-Wheeler aligner v0.7.17, followed by PCR deduplication using Picard v2.9.0. Realignment around small insertions and deletions was performed and then, germline variants were called using HaplotypeCaller using Genome Analysis Toolkit (GATK) v3.5. Variants were then filtered for base sequencing quality score ≥ 30 and annotated using Ensembl Variant Effect Predictor (VEP) and GEMINI v0.19.1.

Variants in *RAD51C* and *RAD51D* were extracted from the annotated variant call files (VCF) for filtering and prioritization (**Figure 3.1-B**). Silent and intronic variants and

those with a minor allele frequency (MAF) ≥ 0.01 in the Genome Aggregation Database of non-cancer population (gnomAD) v2.1.1 (gnomad.broadinstitute.org) [35,36], low coverage (<10 reads) and variant allele frequency (VAF) <0.20 and >0.80 were filtered out [37]. Manual inspection was performed to confirm variants using Integrative Genomics Viewer (IGV) v2.4.10.

We prioritized for further investigation (see **Figure 3.1-B**) LoF variants and missense variants classified as pathogenic, likely-pathogenic or VUS in ClinVar [38,39] and the American College of Medical Genetics and Genomics (ACMG) guidelines [40]. We prioritized variants predicted to overlap conserved residues or be damaging at the RNA or protein level by at least one of several in silico tools selected based on their best performance [41–43]. Briefly, we selected variants having a conserved prediction score ≥ 2.0 by Genomic Evolutionary Rate Profiling v1.0 (GERP++ v1.0) [44], ≥0.2 Phylogenetic *p* value v4.0 of 100 vertebrates (PhyloP 100 way v4.0) and ≥0.4 by PHAST Conservation v4.0 of 100 vertebrates (PhastCons 100 way v4.0) [45]; damaging prediction score ≥ 0.4 by Rare Exome Variant Ensemble Learner v4.0 (REVEL v4.0) [46], Meta-analytic Logistic Regression v4.0 (MetaLR v4.0) [47] and CONsensus DELeteriousness v2.0 (CONDEL v2.0) [48], ≥0.0 by Meta-analytic Support Vector Machine v4.0 (MetaSVM v4.0) [47]; ≥−2.5 by Protein Variation Effect Analyzer v4.0 (PROVEAN v4.0) [49] and ≥20 (Phred score = −10 log10 P) by Combined Annotation Dependent Depletion v1.4 (CADD v1.4) [50]. Prediction performance of these in silico tools was tested based on germline variants submitted to the ClinVar database and classified by ACMG guidelines [41,43]. Variants were predicted to affect splicing if the score was ≥0.4 by two of Database Splicing Consensus Single Nucleotide Variant (dbscSNV) in silico tools [45]: AdaBoost v1.1 (ADA v1.1) or Random Forest v1.1 (RF v1.1); or classified to affect splicing by Maximum Entropy Estimates of Splice Junction Strengths v2.0 (MaxEntScan v2.0) [51] and Human Splicing Finder v3.1 (HSF v3.1) [52]. Prediction performance of these splicing predictor in silico tools was tested on somatic variants submitted to the catalogue of somatic mutations in cancer (COSMIC) database [42].

Candidate variants were verified in PBL DNA by bidirectional Sanger sequencing of PCR products using customized primers (**Table S3.2.**) at the McGill Genome Centre

as previously described [28]. Sequencing chromatograms were visually inspected for variant heterozygosity using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer institute, Amsterdam, The Netherlands).

3.4.3. Investigating Carrier Frequencies of Candidate Variants in FC OC Cases and Controls

Carrier frequencies of candidate variants were determined by genotyping PBL DNA from OC or BC cases from defined FC study groups (**Table S3.1.**) using customized TaqMan assays [53] (**Table S3.2.**). Carriers were verified by bidirectional Sanger sequencing as described above. Corresponding tumour DNA from the index case was genotyped where PBL DNA was not available.

Carrier frequencies of candidate variants were determined in FC controls by surveying 1025 sequencing-based: 433 from Gen3G, 422 from MNI and 170 from CARTaGENE [28,31,33] and 8493 SNP genotyping-based [31] data available from CARTaGENE resources (**Table S3.1.**). Carrier counts were extracted from VCF files or PLINK files. If the variant probes were not represented on the SNP array, pre-phasing and imputation were performed using Eagle2 with the Burrows-Wheeler transformation through Sanger Imputation Services (sanger.ac.uk/tool/sanger-imputation-service/, accessed on 1 February 2019), where Haplotype Reference Consortium release v1.1 (HRC.r1) was used as the reference [54,55].

Fisher's exact test was used to compare carrier or allele frequencies in appropriate OC study groups versus controls using two-tailed *p* values where <0.05 was considered significant. A permutation analysis using Fisher's exact test was also performed of cases and controls to address the possibility that carriers could have been recruited to more than one FC study group (**Table S3.1.**).

3.4.4. Surveying Allele and Carrier Frequencies of Candidate Variants in Genetic Databases of Non-FC Populations

Carrier or allele frequencies of candidate variants were surveyed in genetic data that was derived from cancer-free individuals of European ancestry, as the original founders of FCs of Quebec were of Western European (France) origin [20,21,27,56]. Carrier or

allele frequencies were determined by querying data available from three resources (**Table S3.3.**). Allele frequencies for the non-cancer of non-Finnish European ancestry were extracted from WES or whole genome sequencing (WGS) data deposited in the Genome Aggregation Database (gnomAD) v2.1.1 (gnomad.broadinstitute.org, accessed on 1 October 2021). For comparative purposes, we also extracted data derived from other populations from this resource. Carrier frequencies for 7325 women of European ancestry regardless of family history of cancer were extracted from genetic data from the Fabulous Ladies Over Seventy (FLOSSIES) project (whi.color.com, accessed on 1 October 2021), which included data from a 27-gene panel sequencing study.

Candidate variants were also queried in genetic data from 25,509 OC cases and 40,491 controls using the Ovarian Cancer Association Consortium (OCAC) database (ocac.ccge.medschl.cam.ac.uk/data-projects/, accessed on 15 June 2020). Summary statistics provided included odd ratios (OR_{Log2}) with *p* values comparing all epithelial OC histopathological subtypes with OCAC controls.

For comparison purposes, we also queried *BRCA1* c.4327C>T; p.Arg1443Ter in all of these resources, as this variant is the most frequently reported pathogenic *BRCA1* variant in the FC and European populations [21].

3.4.5. LOH Analysis of RAD51C and RAD51D Loci in OC Tumour DNA from Candidate Variant Carriers

LOH analysis of *RAD51C* and *RAD51D* loci was performed by Sanger sequencing of OC tumour DNA from variant carriers, where possible, using customized primers as described above (**Table S3.2.**). Extracted DNA from fresh-frozen (FF) or histopathological sections from formalin-fixed paraffin-embedded (FFPE) tumour tissues were provided from the RRCancer biobank for DNA extraction based on the manufacturer's instructions (Promega, Canada). Sequencing chromatograms were visually inspected for complete or partial loss of the wild-type *RAD51C* or *RAD51D* alleles from carriers using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer institute, Amsterdam, The Netherlands).

3.4.6. RNA Extraction and Reverse Transcription Analyses of RAD51C

An Epstein–Barr virus transformed lymphoblastoid cell line (LCL) was established from PBLs from *RAD51C* c.705G>T carriers and non-carriers of this variant as previously described [57]. Approximately 5,000,000 cells were treated with 28 µg/mL of cycloheximide or DMSO for 3 h. RNA was extracted from cell pellets treated with 1000 µL of TRIzol (Invitrogen, Canada) for reverse transcription [58]. cDNA was amplified and purified for Sanger sequencing using customized primers as described above (**Table S3.4.**). Sequencing chromatograms were visually inspected for splicing impact using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer institute, Amsterdam, The Netherlands).

3.4.7. Cell Lines

We used three cell lines in our assays, HeLa (cervical carcinoma), U2OS (sarcoma) and OVCAR-3 (epithelial ovarian adenocarcinoma). HeLa cells were obtained from American Type Culture Collection (atcc.org) and maintained in Dulbecco's Modified Eagle Media supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, at 37 °C, 5% CO_2, and 20% O_2. OVCAR-3 cells were grown in RPMI supplemented with 0.01 mg/mL bovine insulin and 20% foetal bovine serum, at 37°C, 5% CO_2. U2OS cells RAD51D knock-out (KO) [59] were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (U2OS#18-RAD51D-4*, DSMZ*, Braunschweig, Germany) maintained in Dulbecco's Modified Eagle Media supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, at 37 °C, 5% CO_2, and 20% O_2. OVCAR-3 cells were a kind gift from Dimcho Batchvarov (CHU de Québec).

3.4.8. Complementation Assays and siRNA Transfections

For in cellulo experiments in HeLa and OVCAR-3 cells, the RAD51D p.Ser46Cys protein variant was obtained via site-directed mutagenesis using the Q5 Site-Directed Mutagenesis Kit (NEB) (New England biolabs, Canada) using the pcDNA3-RAD51D wild-type (WT) as a template and primers listed on **Table S3.4.** The mammalian expression vector pcDNA3-RAD51D that was used as a template had been previously modified for RAD51D expression with a FLAG tag at the N-terminus and for resistance

to RAD51D siRNA with the Q5 Site-Directed Mutagenesis Kit (NEB) and primers listed on **Table S3.4.** The siRNA target sequence used to silence RAD51D was siRAD51D #7 GGCCAAAUCUUCCCGACAGdTdT and the non-specific siRNA used as control was UUCGAACGUGUCACGUCAAdTdT.

Approximately 240,000 cells were seeded into one well of a six-well plate before being double transfected 24 and 48 h later with 50 nM control or RAD51D siRNA using Lipofectamine RNAiMAX (Invitrogen, Canada). For Hela, cells were then complemented by transfection of 800 ng of the pcDNA3 empty vector or the indicated siRNA-resistant FLAG-tagged RAD51D construct using Lipofectamine 2000 (Invitrogen, Canada) for four hours. Transient transfections in OVCAR3 cells were performed with 300 or 700 ng of the pcDNA3 empty vector or the indicated siRNA-resistant FLAG-tagged RAD51D construct using Lipofectamine 3000 (Invitrogen, Canada) for four hours according to the manufacturer's protocol. U2OS RAD51D KO cells were stably complemented using the AAVS1 integration system [60]. The AAVS1 RAD51D WT or p.Ser46Cys constructs were generated by amplification using the pcDNA3 plasmids previously described as a template and primers listed on **Table S3.4.**; both products were cloned into the AAVS1 vector in NotI/PspXI sites. Briefly, cells were transfected with the 4 µg of the AAVS1 construct containing either the WT or the RAD51D p.Ser46Cys variant, along with the 0.4 µg of the pZFN plasmid, using Lipofectamine 2000 (Invitrogen) for 4 h. 24 h later, transfected cells were selected with G418 for 7 days. To confirm genomic integration, genomic DNA was extracted from stable cells using PureLink Genomic DNA Mini Kit (Invitrogen) and used as a template to amplify the integrated cDNA in the AAVS1 locus using primers from **Table S3.4.** Complementation was then confirmed by Sanger sequencing.

3.4.9. Olaparib and Talazoparib Sensitivity Assays

PARP inhibitors, olaparib and talazoparib, sensitivity assay and cell imaging were performed as described previously [61]. Cells were treated for four days with concentrations ranging from 0 (DMSO) to 2.5 µM of olaparib or 0 (DMSO) to 40 nM of talazoparib. Cell viability was expressed as percentage of survival in olaparib-treated or talazoparib-treated cells relative to vehicle (DMSO)-treated cells. Results represent the

mean ± standard error of the mean (SEM) of at least three independent experiments, each performed in triplicate.

3.4.10. Immunofluorescence Analysis

U2OS RAD51D cells stably complemented with either RAD51D WT or the p.Ser46Cys were seeded into Corning 96-Well Half Area High Content Imaging Film Bottom Microplate at 7000 cells per well. Then, 18h later, cells were irradiated with 5 Gray and processed for immunofluorescence 4 h post-irradiation. Unless otherwise stated, all immunofluorescence dilutions were prepared in PBS and incubations performed at room temperature with intervening washes in PBS. Cell fixation was carried out by incubation with 4% paraformaldehyde for 10 min followed by 100% ice-cold methanol for 5 min at −20◦C. Cells were permeabilised in 0.2% Triton X-100 for 5 min followed by a quenching step using 0.1% sodium borohydride for 5 min. After blocking for 1 h in a solution containing 10% goat serum and 1% BSA, cells were incubated for 1 h with primary antibody anti-RAD51 (1:5000, Bioacademia #70-001) or anti-phospho-Histone H2A.X (Ser139) (1:5000, Millipore, #05-636), combined with anti-Geminin (1:7000, Abcam #ab104306 or Proteintech #10802-1-AP) all diluted in 1% BSA. Secondary antibody labelling used Alexa Fluor 488 goat anti-rabbit (Invitrogen, #A-11008) and Alexa Fluor 647 goat anti-mouse (Invitrogen, #A21235) or Alexa Fluor 488 goat anti-mouse (Invitrogen, #A-11001) and Alexa Fluor 647 goat anti-rabbit (Invitrogen, #A21244), diluted at 1:1000 in 1% BSA for 1 h. Nuclei were stained for 10 min with 1 mg/mL 4,6-diamidino-2-phenylindole (DAPI). Z-stack images were acquired on a ZEISS Celldiscoverer 7 automated microscope using a 50× water immersion objective and analysed for RAD51 or gH2AX foci formation with ZEN Blue software 3.2 (ZEISS). Data from three independent trials were analysed for outliers using the ROUT method (Q = 1.0%) in GraphPad Prism v8.0 and the remaining were reported in a violin plot.

3.4.11. Protein Expression and Immunoblotting Analyses of RAD51D

Total soluble protein extraction and immunoblotting were performed as previously described [62]. For RAD51D detection, a polyclonal antibody (#ab202063, Abcam, US) was used at a 1:1000 dilution. Mouse monoclonal anti-vinculin (#V9131, Sigma, US) at

1:200,000 dilution was used as the loading control. Horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse at 1:10,000 dilution (Jackson Immuno Research, US) was used as secondary antibodies.

3.5. Results

3.5.1. Identification and Characteristics of Candidate Variants

By extracting all variants located in protein encoding and splice-site regions of *RAD51C* and *RAD51D* in WES data, we identified a total of 8 variants in 20 familial cases and 16 variants in 53 sporadic cases of OC (**Figure 3.1.** and **Table S3.5.**). From this list, we identified five candidate variants in these genes that fulfilled our selection criteria (**Figure 3.1-B**): two missense variants in *RAD51C* c.414G>C; p.Leu138Phe and c.705G>T; p.Lys235Asn, two missense variants in *RAD51D* c.137C>G; p.Ser46Cys and c.620C>T; p.Ser207Leu and a nonsense variant *RAD51D* c.694C>T; p.Arg232Ter. The variants were identified in nine OC cases: 17.6% (3/17) of OC families and 11.3% (6/53) of sporadic early-onset cases (**Table 3.1.**). Our results include the identification of newly reported variants in FCs: two carriers of *RAD51C* c.414G>C; p.Leu138Phe, one of *RAD51C* c.705G>T; p.Lys235Asn, one of *RAD51D* c.137C>G; p.Ser46Cys and one of *RAD51D* c.694C>T; p.Arg232Ter. In addition, our results include four carriers of *RAD51D* c.620C>T; p.Ser207Leu, a variant previously reported to occur in more than one FC OC case by our group [28].

Our candidate variants are rare in various cancer-free populations of non-FC and European ancestry based on surveying available genetic data (**Tables 3.1.** and **S3.5.**). All candidate variants have a MAF ≤ 0.0001 in the non-cancer population represented in the gnomAD database and in cancer-free women in the FLOSSIES database.

All variants were predicted to affect highly conserved loci and the four missense variants to be damaging by at least one of our in silico tools that we selected based on their best performance (**Table 3.1.**). Four different in silico tools also predicted that the *RAD51C* c.705G>T; p.Lys235Asn located at the 5′ splice-donor site would affect transcript splicing.

The ClinVar database and/or ACMG guidelines classified *RAD51C* c.414G>C; p.Leu138Phe and c.705G>T; p.Lys235Asn and *RAD51D* c.694C>T; p.Arg232Ter as

likely pathogenic or pathogenic. In contrast, there were conflicting classifications reported for *RAD51D* c.137C>G; p.Ser46Cys, such as VUS and likely benign in the ClinVar database and VUS by ACMG guidelines and for *RAD51D* c.620C>T; p.Ser207Leu, such as pathogenic/likely pathogenic in the ClinVar database and VUS by ACMG guidelines.

None of the five candidate variants were found to co-occur in the nine carriers of these variants. We also reviewed WES data of these nine carriers for the presence of pathogenic variants in any of the known risk genes for OC based on the National Comprehensive Cancer Network (NCCN) for clinical practice in oncology guidelines (version 2021–Genetic/Familial High-Risk Assessment: Breast, Ovarian and Pancreatic): *BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *MSH6*, *PMS2*, *BRIP1*, *RAD51C*, *RAD51D*, *PALB2*, *ATM* or *STK11*. All carriers were found not to carry a pathogenic or likely pathogenic variant in any of these genes based on ClinVar or ACMG guidelines, with the exception of the *RAD51D* c.694C>T; p.Arg232Ter carrier who also harboured a pathogenic variant in *BRCA1* c.1462dupA; p.Thr488AsnfsTer2. Interestingly, this known *BRCA1* pathogenic variant has not been previously reported in the FC population.

3.5.2. Carrier Frequency of Candidate Variants in OC Cases and Cancer-Free Controls of FC Ancestry

We compared the carrier frequencies of our candidate variants in different FC groups comprised of cancer cases, regardless of *BRCA1* or *BRCA2* pathogenic variant status, and cancer-free controls (**Table 3.2.**). Pair-wise comparisons of carrier frequencies were performed using data from each cancer group and sequencing-based controls (see **Table S3.1.**). The highest overall carrier frequency was among carriers of *RAD51D* c.620C>T found in the sporadic group. Frequencies of this variant ranged from 2.3% (1/44) in OC families having at least two OC cases to 3.4% (15/438) in sporadic cases. Notably, all carriers were among the pathogenic *BRCA1* or *BRCA2* variant-negative cases as determined by previous studies which included whole gene or targeted genotyping of FC variants in these study groups (see **Table S3.1.**). The carriers in the sporadic cases included the previously identified carriers of this variant (3.8%; 13/341) [28]. In contrast, the carrier frequencies of each of the other variants were lower

Table 3.1. Characteristics of candidate variants identified in *RAD51C* and *RAD51D*.

Gene	***RAD51C***	***RAD51C***	***RAD51D***	***RAD51D***	***RAD51D***
Genomic features (GRCh37/hg19)					
RefSeq transcript no.	NM_058216.3	NM_058216.3	NM_002878	NM_002878	NM_002878
Genome change	g.56774063G>C	g.56780690G>T	g.33446137G>C	g.33430520G>A	g.33430317G>A
Coding change	c.414G>C	c.705G>T	c.137C>G	c.620C>T	c.694C>T
Protein change	p.Leu138Phe	p.Lys235Asn	p.Ser46Cys	p.Ser207Leu	p.Arg232Ter
Number of carriers discovered (Phase I)					
Familial OC cases (n = 20)	1	0	0	2	0
Sporadic OC early-onset cases (n = 53)	1	1	1	2	1
Allele frequencies in gnomAD[1]					
Non-Finnish European	0.00001 (1/102,736)	0.00001 (1/102,610)	0.0001 (16/118,138)	0.0001 (6/118,136)	0.00003 (4/126,578)
Carrier frequencies in FLOSSIES [2]					
European	0 (0/7325)	0 (0/7325)	0.0002 (2/7325)	0.0003 (3/7325)	0.0001 (1/7325)
Clinical classification[3]					
ClinVar (number of submissions)	Pathogenic/Likely pathogenic (7)	Conflicting (7): Likely pathogenic (1); Uncertain significance (6)	Conflicting (8): Uncertain significance (7); Likely benign (1)	Conflicting (11): Pathogenic (2); Likely pathogenic (6); Uncertain significance (3)	Pathogenic (15)
ACMG guidelines (classification codes)	Likely pathogenic	Pathogenic (PS3; PM2)	Uncertain significance	Uncertain significance	Pathogenic

	(PS1; PM2; PP3; PP5)		(PM2; PP3)	(PS3; M2; PP3)	(PVS1; PM2; PP3; PP5)
Predictions by in silico tools[4]					
GERP++ v1.0	Conserved	Conserved	Conserved	Conserved	Conserved
PhyloP 100 way v4.0	Conserved	Conserved	Conserved	Conserved	Conserved
PhastCons 100 way v4.0	Conserved	Conserved	Conserved	Conserved	Conserved
REVEL v4.0	Pathogenic	Benign	Pathogenic	Pathogenic	-
MetaLR v4.0	Tolerated	Tolerated	Tolerated	Damaging	-
MetaSVM v4.0	Tolerated	Tolerated	Tolerated	Damaging	-
CONDEL v2.0	Damaging	Tolerated	Damaging	Damaging	-
PROVEAN v4.0	Damaging	Tolerated	Damaging	Damaging	-
CADD v1.4	Damaging	Damaging	Damaging	Damaging	Damaging
ADA v1.1	-	Affecting splicing	-	-	-
RF v1.1	-	Affecting splicing	-	-	-
HSF v3.1	-	Affecting splicing	-	-	-
MaxEntScan v2.0	-	Affecting splicing	-	-	-

[1] Allele frequencies in non-cancer controls from gnomAD v2.1.1 database (gnomad.broadinstitute.org). Allele frequencies in non-cancer controls from different populations from gnomAD v2.1.1 database are presented in **Table S3.5**. [2] Carrier frequencies from non-cancer controls from Fabulous Ladies Over Seventy (FLOSSIES) database (whi.color.com/about) (see **Table S3.4.**). [3] Clinical classifications from ClinVar (ncbi.nlm.nih.gov/clinvar/) [38,39] and American College of Medical Genetics and Genomics (ACMG) guidelines and associated codes [40] based on last revision reviewed in October 2021 as PS1: Pathogenic Strong Level 1; PS3; Pathogenic Strong Level 3; PM2: Pathogenic Moderate Level 2; PP3: Pathogenic Supporting Level 3; PP5: Pathogenic Supporting Level 5; and PVS1: Pathogenic Very Strong Level 1. [4] Details of in silico tools applied: ADA v1.1: AdaBoost v1.1; CADD v1.4: Combined Annotation Dependent Depletion v1.4; CONDEL v2.0: CONsensus DELeteriousness

v2.0; GERP++ v1.0: Genomic Evolutionary Rate Profiling v1.0; HSF v3.1; Human Splicing Finder v3.1; MaxEntScan v2.0: Maximum Entropy Estimates of Splice Junction Strengths v2.0; MetaLR v4.0: Meta-analytic Logistic Regression v4.0; MetaSVM v4.0: Meta-analytic Support Vector Machine v4.0; PhyloP 100 way v4.0: phylogenetic *p* value v4.0 of 100 vertebrates; PhastCons 100 way v4.0: PHAST Conservation v4.0 of 100 vertebrates; PROVEAN v4.0: Protein Variation Effect Analyzer v4.0; RF v1.1: Random Forest v1.1; REVEL v4.0: Rare Exome Variant Ensemble Learner v4.0. OC: ovarian cancer; RefSeq: reference sequence; and (-): Not applicable/available.

in the cancer groups. The carrier frequencies of these variants ranged from 0% to 2.3% (1/44) in OC families harbouring *RAD51C* c.414G>C, where the carrier was from a pathogenic *BRCA1* or *BRCA2* variant-negative family, and from 0% to 0.2% (1/438) for those harbouring *RAD51C* c.705G>T, *RAD51D* c.137C>G or c.694C>T variants. None of the index OC or BC cases from 56 HBOC families were found to harbour any of our candidate variants. Our targeted genotyping assays or review of available WES data revealed that none of the carriers identified in the cancer study groups (**Table 3.2.**) also carried another one of our *RAD51C* or *RAD51D* candidate variants.

Table 3.2. Carrier frequency of candidate variants in French Canadian study groups and comparison of cancer cases to controls.

Variant	Study Groups	Cancer Case Tested	Number of Participants or (Families) per Group	Number of Carriers (%)	*p* Value [1]
***RAD51C* c.414G>C**	OC families	OC	49 (44)	1/44 (2.3)	0.081
	HBOC families	OC or BC	56 (56)	0	-
	Sporadic OC cases	OC	438	0	-
	Sequencing-based controls	-	1025	1/1025 (0.1)	-
***RAD51C* c.705G>T**	OC families	OC	49 (44)	0	-
	HBOC families	OC or BC	56 (56)	0	-
	Sporadic OC cases	OC	438	1/438 (0.2)	0.299
	Sequencing-based controls	-	1025	0	-
***RAD51D* c.137C>G**	OC families	OC	49 (44)	0	-
	HBOC families	OC or BC	56 (56)	0	-
	Sporadic OC cases	OC	438	1/438 (0.2) [2]	0.299
	Sequencing-based controls	-	1025	0	-
***RAD51D* c.694C>T**	OC families	OC	49 (44)	0	-
	HBOC families	OC or BC	56 (56)	0	-
	Sporadic OC cases	OC	438	1/438 (0.2) [2]	0.299
	Sequencing-based controls	-	1025	0	-

***RAD51D* c.620C>T**	OC families	OC	49 (44)	1/44 (2.3)	0.081
	HBOC families	OC or BC	56 (56)	0	-
	Sporadic OC cases	OC	438	15/438 (3.4)[3]	<0.0001
	Sequencing-based controls	-	1025	1/1025 (0.1)	-

[1] Two-tailed *p* values calculated using Fisher's exact test in pair-wise comparisons between carriers in cancer study groups and controls; not adjusted for multiple testing. [2] Carriers known to also have been part of the sporadic early-onset OC cases phase I study group (see **Table S3.6.**). [3] Thirteen of 15 *RAD51D* c.620C>T carriers were previously reported [28] (see **Table S3.6.**). BC: Breast cancer; HBOC: Hereditary breast and ovarian cancer syndrome; OC: Ovarian cancer; and (-): Not applicable.

It was not possible to perform pair-wise comparisons to further assess carrier frequency of our variants in cancer study groups and genotyping-based controls. No carriers of *RAD51C* c.414G>C or *RAD51D* c.620C>T were identified in the genotyping-based data of 8493 population-matched controls. Carriers of *RAD51C* c.705G>T, *RAD51D* c.137C>G or *RAD51D* c.694C>T could not be identified in the same data and were not available in the HRC.r1 haplotype reference panel used for imputation from SNP array data.

Although the cancer study groups were independently derived for previous research purposes [21,23,24,34,63], we cannot exclude the possibility that individuals were recruited to multiple study groups. Based on the unique RRCancer biobank sample reference number, we are only aware of nine samples where the same case was included in two different study groups (**Figure S3.1.**). We therefore performed a permutation analysis with 5000 random allocations of the observed variants to the participants across the three cancer groups (44 OC families, 56 HBOC families and 438 sporadic OC cases) and the two control groups (1025 sequencing-based and 8493 genotyping-based controls) investigated this study. When compared to sequencing-based controls, the permutation analysis presented evidence for a higher variant rate among all cases ($p = 0.026$), OC families ($p = 0.015$) and all families ($p = 0.026$). When examining only *RAD51D* c.620C>T, which was captured in data from both sequencing-

based and genotyping-based controls, permutation analysis provided evidence for a higher frequency of this variant in all cancer cases versus all controls (p = 0.0098) and in all familial cases versus all controls (p = 0.014). The permutation analysis also allowed us to estimate the family-wise error rate for all tests performed: in 6.7% of the permutations, we found that at least one of the five tests demonstrated significance at p < 0.05, reflecting a type-1 error rate of potential concern. However, our permutation analysis also demonstrated that it was highly unlikely for all five comparisons to result in a naïve p-value <0.05 simultaneously (permutation study p = 0.0002).

3.5.3. Clinico-Pathological Characteristics of OC Variant Carriers

The histopathological and clinical characteristics available for the 6 *RAD51C* and 28 *RAD51D* variant carriers are shown in **Table S3.6.**, which also includes known personal history of cancer. The pedigrees of selected carriers are shown in **Figure S3.2.**, anonymized to only show information relevant to this study to protect the identity of participants. Thirteen of 28 *RAD51D* c.620C>T carriers from a previous study were also included for comparative purposes as their associated clinical features had not been reported [28]. Features of carriers of *RAD51C* c.705G>T (n = 2) and *RAD51D* c.137C>G (n = 1) and c.620C>T (n = 7) that were identified by targeted screening of an additional 538 cases of OC of FC ancestry were also included in **Table S3.6.**

Most OC carriers of our candidate variants had HGSC (31/34), which is the most common subtype of epithelial OC [64]. Three other carriers had either a high-grade endometrioid adenocarcinoma, serous carcinoma of unknown grade or OC of undisclosed histopathology. A query of the OCAC data, which only revealed summary statistics for one of our candidates, showed statistical differences in the frequency of *RAD51D* c.620C>T carriers having HGSC (OR_{Log2} = 17.2; p = 0.00001) versus controls (**Table S3.7.**). This observation is consistent with our query of *BRCA1* c.4327C>T in OCAC data (OR_{Log2} = 1.211; p = 0.009051), the most common pathogenic FC OC risk allele as a comparator, where we found statistical differences in the frequency of carriers of this *BRCA1* variant having the high-grade serous subtype OC versus controls.

The average age of OC diagnosis in carriers of 58.5 years (median 59 years [age range = 42–77; SD ± 9.0 years]) was comparable to the average age of OC diagnosis in

the general population being 60 years of age [28]. Fifty-three percent (18/34) of carriers were diagnosed before the age of 60 years, where 21% (7/34) were diagnosed before the age of 50 years.

Given the high frequency of *RAD51D* c.620C>T carriers in our OC cases, it was possible to compare clinical data of carriers (15/438) of this *RAD51D* variant with carriers (15/438) [21,28] of a frequently occurring variant in *BRCA1* c.4327C>T [21,23,24,34,65], previously reported in our investigation of the same sporadic OC study group [34]. The average and median ages at diagnosis of *RAD51D* variant carriers was approximately 59 years (age range = 46–74; SD ± 8.4 years). This was older than the average and median ages of diagnosis of 54 years observed in *BRCA1* variant carriers (age range = 36–76; SD ± 11 years; $p = 0.15$; 95% CI: −1.96 to 12.49; two tailed *p* value, unpaired *t*-test). The average survival among the 15 *RAD51D* c.620C>T carriers was 81.9 months (median 69 months [range = 1–195 months]) which was longer than the average survival of 67.1 months among *BRCA1* c.4327C>T carriers, though not significantly different (median 52 months [range = 10–168 months]; $p = 0.46$; 95% CI: −25.86 to 55.33, two-tailed *p* value, unpaired *t*-test). There were 46.7% (7/15) of *RAD51D* c.620C>T carriers and 60% (9/15) of *BRCA1* c.4327C>T carriers who had died of OC.

3.5.4. LOH Analyses of RAD51C and RAD51D Loci in OC Tumour DNA from Candidate Variant Carriers

Evidence of partial or complete loss of the wild-type allele was observed in tumour DNA from at least one carrier of each type of missense candidate variant (**Table 3.3.**) as tumour DNA was not available for all variant carriers. Although our assays were not performed in DNA samples extracted from sections enriched for cancer cells, in five cases harbouring *RAD51D* c.137C>G or c.620C>T (PT0058, PT0071, PT0075, PT0076 and PT0077), there was clear evidence of loss wild-type allele in the analysis of tumour DNA extracted from FFPE. These findings suggest that partial loss or allelic imbalance observed with some samples may be an indication of contaminating normal stromal cells, although OC tumour specimens are often abundant in cancer cells [66]. Interestingly, only the *RAD51D* c.620C>T germline allele was detectable in both OC

tumours from a bilateral OC case by Sanger sequencing of tumour DNA (**Figure 3.2.**). This observation suggests the possibility that somatic loss of the wild-type allele preceded clonal expansion in the tumourigenesis of OC in this carrier.

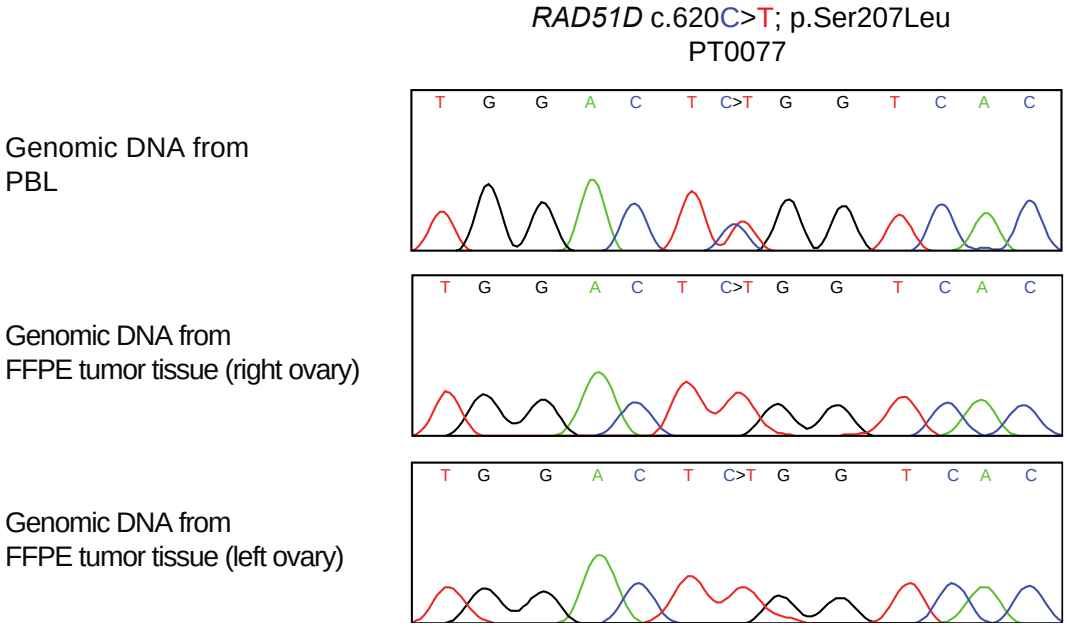

Figure 3.2. Loss of heterozygosity analysis of a *RAD51D* c.620C>T carrier.

Sanger sequencing chromatograms showing evidence of a complete loss of the wild-type variant in genomic DNA from formalin-fixed paraffin embedded (FFPE) tumour tissues from both ovaries.

3.5.5. In Vitro Investigation of Aberrant Splicing of RAD51C c.705G>T

We established *RAD51C* c.705G>T carrier- and non-carrier-derived LCLs and performed RT-PCR on the extracted RNA to determine if the genomic position of the variant affected splicing consistent with predictions based on the application of our selected in silico tools (**Table 3.1.**). RT-PCR analyses showed two different size bands from the c.705G>T carrier-derived LCLs but not in controls (**Figure 3.3-A**), suggesting that this variant affected splicing of the transcript. Although we could not verify the presence of exon 4 in the non-carrier as cDNA no longer available, Sanger sequencing verified the absence of the entire exon four in the variant carrier-derived cDNA (**Figure 3.3-B-C**), suggesting that exon skipping had occurred.

Table 3.3. Loss of heterozygosity analyses of tumour DNA from ovarian cancer carriers by Sanger sequencing.

Carrier ID[1]	Gene	Coding Change[2]	Protein Change	Germline Status	Laterality of Disease	LOH Analyses of Available DNA from Fresh Frozen Tumour			LOH Analyses of Available DNA from Formalin-Fixed Paraffin-Embedded Tumour	
						Right ovary	Left ovary	Laterality unknown or alternative tissue	Right ovary	Left ovary
PT0095	*RAD51C*	c.414G>C	p.Leu138Phe	Heterozygous	Unilateral (Left)	-	-	-	-	-
PT0094	*RAD51C*	c.414G>C	p.Leu138Phe	Heterozygous	Bilateral	-	-	Partial loss in ascites	-	-
PT0124	*RAD51C*	c.705G>T	p.Lys235Asn	Heterozygous	Bilateral	Partial loss	-	-	-	-
PT0125	*RAD51C*	c.705G>T	p.Lys235Asn	Heterozygous	Bilateral	-	Complete loss	-	-	-
PT0126[3]	*RAD51C*	c.705G>T	p.Lys235Asn	Heterozygous	Bilateral	Heterozygous	-	-	-	-
PT0127	*RAD51C*	c.705G>T	p.Lys235Asn	Heterozygous	Unknown	-	-	-	-	-
PT0143	*RAD51D*	c.694C>T	p.Arg232Ter	Heterozygous	Bilateral	-	-	-	-	-
PT0058	*RAD51D*	c.137C>G	p.Ser46Cys	Heterozygous	Bilateral	-	-	Heterozygous	Partial loss	-
PT0145	*RAD51D*	c.137C>G	p.Ser46Cys	Heterozygous	Bilateral	Partial loss	-	-	-	-
PT0080	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	Partial loss in omentum	-	-

PT0071	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	Partial loss	-	-	Partial loss	-
PT0073	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Unilateral (Left)	-	-	-	-	-
PT0090	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	-	-	-
PT0078	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	-	-	-
PT0079	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Unilateral (Left)	-	-	-	-	-
PT0089	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	-	-	-
PT0059	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	Complete loss in ovary	-	-
PT0065[3]	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	Heterozygous in ovary	-	-
PT0075[3]	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Unilateral (Right)	Partial loss	-	-	Complete loss	-
PT0076	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	-	Complete loss	Partial loss
PT0077	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	-	Complete loss	Complete loss
PT0074[3]	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	Partial loss	-	-	-	-
PT0144	*RAD51D*	c.620C>T	p.Ser207Leu	Heterozygous	Bilateral	-	-	Heterozygous	-	-

[1] The 13 carriers with high-grade serous ovarian carcinomas (HGSC) previously reported are not included in this table. [2] Transcripts of *RAD51C* (NM_058216.3) and *RAD51D* (NM_002878.4) are based on the NCBI Reference Sequence (RefSeq) database (ncbi.nlm.nih.gov/refseq/). [3] The DNA was extracted from tumour samples post-chemotherapy treatment. (-): Tumour DNA not available or failed analyses.

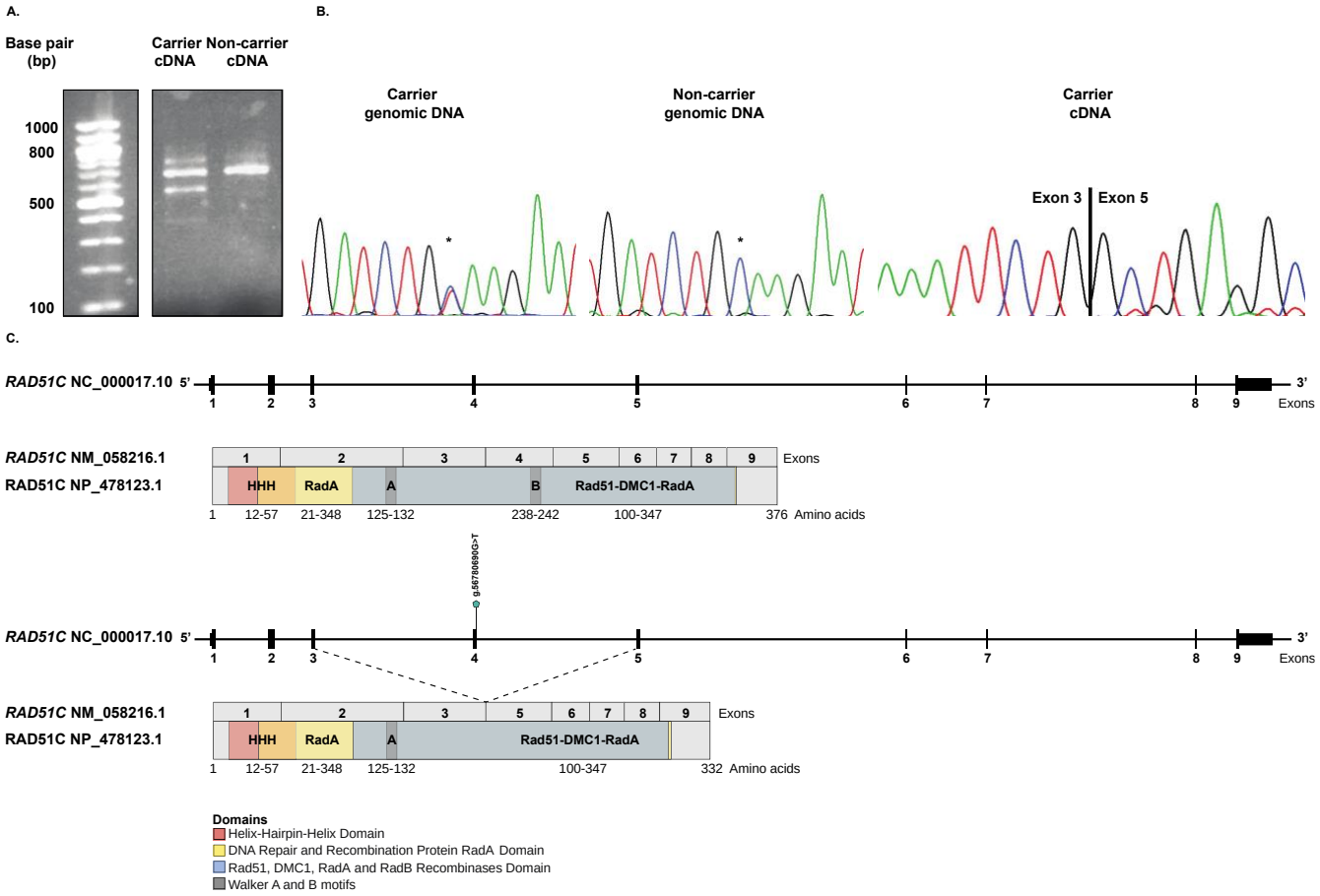

Figure 3.3. *RAD51C* c.705G>T effect on transcript splicing in carrier- and non-carrier-derived lymphoblastoid cell lines.

(A) Agarose gel of cDNA analysis showing different sized bands from carrier- compared to non-carrier-derived LCLs (see **Figure S3.3.**); (B) Sanger sequencing chromatograms of genomic and cDNA from carrier-derived LCLs, confirming the variant status as indicated with * in genomic DNA and showing skipping of exon four in cDNA; and (C) The upper panel depicting the wild-type *RAD51C* at the genomic, mRNA and protein level whereas the lower panel depicting the predicted effect of *RAD51C* c.705G>T at mRNA and protein level, resulting in skipping of exon four (44 amino acids) annotated according to genomic (NC_000017.10), mRNA (NM_058216.1) and protein (NP_478123.1) NCBI Reference Sequence (RefSeq) Database (ncbi.nlm.nih.gov/refseq/, accessed on 1 October 2021).

3.5.6. In Cellulo Investigation of RAD51D p.Ser46Cys

We performed in cellulo assays of RAD51D p.Ser46Cys due to conflicting reports of its clinical significance and paucity of data concerning its effect on biological function. We selected this candidate variant for further study as the biological impact of our other remaining missense candidate variants, RAD51C p.Leu138Phe [5] and RAD51D p.Ser207Leu [28] have been reported, or have been inferred as biologically relevant as described above for the effects of splicing on *RAD51C* c.705G>T.

Given the role of RAD51D in HR function, we investigated sensitivity to the PARP inhibitor, olaparib, taking advantage of the synthetic lethal interaction between loss of HR function and PARP inhibition [62]. Using HeLa cells, RAD51D-knock-down cells were more sensitive to olaparib (**Figure S3.4-A**). Complementation with RAD51D WT siRNA resistant construct restored sensitivity to endogenous levels, while RAD51D p.Ser46Cys siRNA resistant construct failed to rescue the viability of RAD51D knock-down cells, showing olaparib sensitivity similar to cells complemented with the empty vector.

Immunoblotting 24 h post-transfection showed that RAD51D p.Ser46Cys protein was weakly expressed compared to WT (**Figure S3.4-B**). As the expression of RAD51D p.Ser46Cys was lower than the WT, we then investigated if the reduced expression of the variant protein could be due to protein instability by examining the protein's half-life in RAD51D knock-down cells transfected with either FLAG-RAD51D WT or p.Se46Cys. Cells were then exposed to cycloheximide (CHX) to inhibit protein synthesis and pellets for protein extraction were collected at the indicated time points. Over the time course RAD51D protein levels were reduced for both isoforms, however, the effect was more pronounced in cells expressing the p.Ser46Cys variant when compared to the WT (**Figure S3.4-C**). RAD51D WT protein starts to reduce at after six hours, while the RAD51D p.Ser46Cys protein is hardly visible at four hours (**Figure S3.4-C**). We were able to recapitulate our findings in an OC cell line background (**Figure S3.5.**). In OVCAR-3 cells, the p.Ser46Cys variant protein is also expressed at a reduced level. These observations suggest that RAD51D p.Ser46Cys is unstable and affects cellular sensitivity to olaparib.

To further investigate the functionality of the p.Ser46Cys variant and eliminate the effect of endogenous RAD51D, we used U2OS RAD51D KO cells stably complemented with either the WT or the p.Ser46Cys variant using the AAVS1 genomic editing system (**Figure 3.4-A**) [59,60]. Although RAD51D KO cells were successfully complemented with either WT and p.Ser46Cys, as confirmed by Sanger sequencing, the p.Ser46Cys variant was also weakly expressed in this cell line when compared to the WT (**Figure 3.4-B-C**).This is in agreement with the observation that U2OS RAD51D cells harbouring the p.Ser46Cys variant failed to complement survival when exposed to olaparib and talazoparib (**Figure 3.4-D-E**).

RAD51D is required to facilitate RAD51 filament formation and proper repair of damage-induced double-strand breaks [67]. Therefore, we evaluated both RAD51 and γH2AX foci formation after treatment with 5 Gray of ionizing radiation in S/G2-cells (**Figure S3.6.**). As expected, a decrease in the mean number of RAD51 foci per cell was observed in RAD51D deficient cells and expression of RAD51D WT partially rescued this phenotype, while rescue was less obvious in cells expressing the p.Ser46Cys (**Figure S3.6-A-B**). Moreover, after ionizing radiation, the p.Ser46Cys variant also exhibited elevated levels of γH2AX foci, while RAD51D WT cells were able to rescue the increased γH2AX foci formation observed in the RAD51D deficient cells (**Figure S3.6-C-D**). Altogether, these results indicate an impaired HR functionality leading to increased DNA double-strand breaks in cells expressing the RAD51D p.Ser46Cys variant.

3.6. Discussion

Our WES analysis of 73 familial and sporadic early-onset OC FCs of Quebec identified five candidate variants in *RAD51C* and *RAD51D*. The genetic analyses of additional FC OC study groups confirmed that *RAD51D* c.620C>T, previously reported in sporadic OC cases by our group [28], occurs at a high frequency in FCs with this disease. This observation is likely due to the unique genetic architecture of FCs of Quebec that has been attributed to common founders of this population [20–22]. OC cases harbouring other variants were found once for the nonsense *RAD51D* c.694C>T and twice for the missense *RAD51D* c.137C>G or *RAD51C* c.414G>C, suggesting that FCs are more genetically heterogeneouspopulation than other populations with common founders

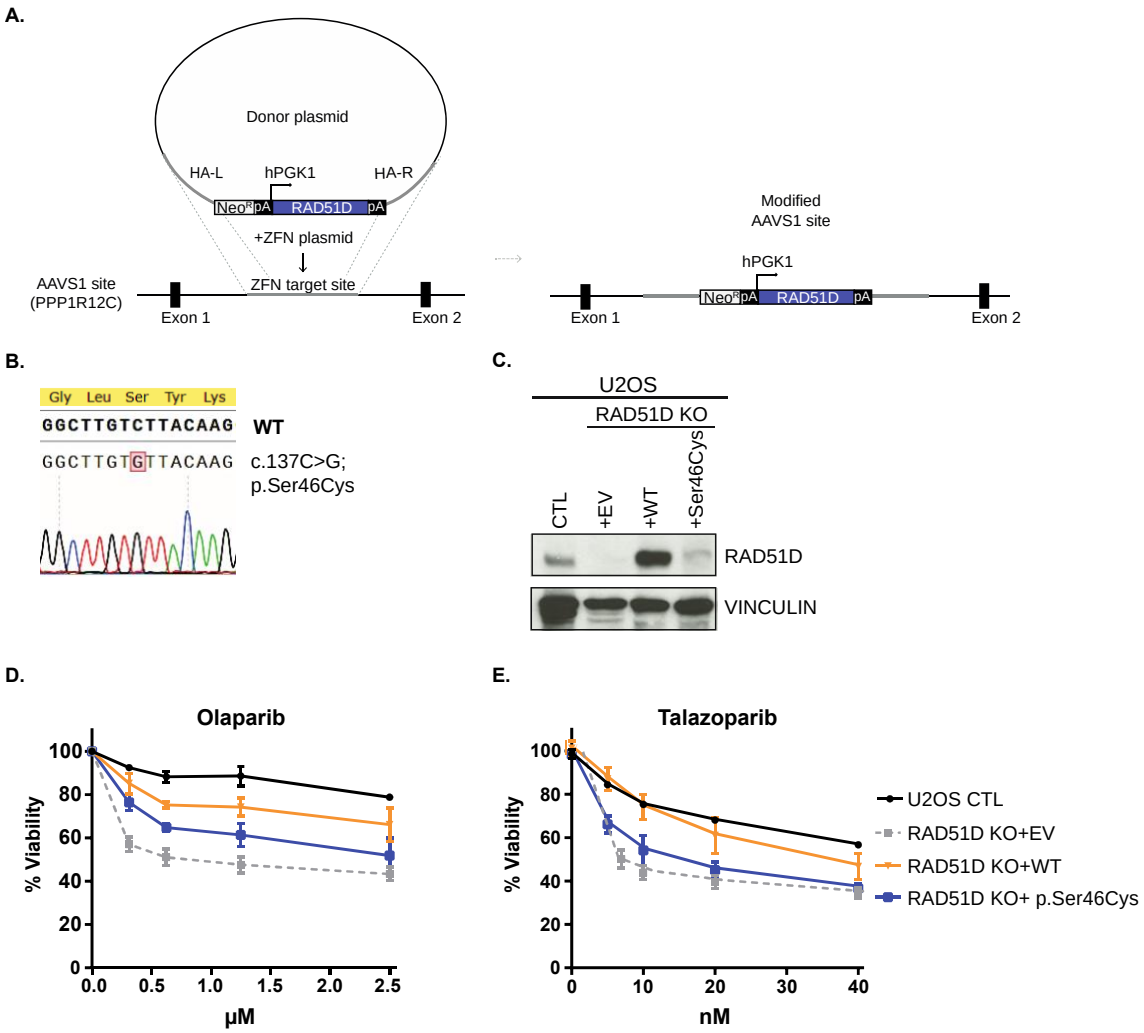

Figure 3.4. The RAD51D p.Ser46Cys variant impairs protein stability and function in U2OS RAD51D knock-out cells.

(A) Scheme representing the AAVS1 genomic integration system used to complement the RAD51D KO U2OS cell line. (B) U2OS RAD51D KO cells complemented with the AAVS1 system were confirmed by Sanger sequencing. (C) Western blots of U2OS RAD51D KO cells stably complemented with wild-type (WT) or the p.Ser46Cys variant (see **Figure S3.7.**); CTL was used as non-edited control and Vinculin was used as a loading control. (D,E) Survival curves of U2OS RAD51D KO cells stably complemented with the WT RAD51D, the RAD51D p.Ser46Cys variant or empty vector (EV) and plated in triplicate in a 96 well plate. Cell viability was monitored following (D) olaparib or (E) talazoparib treatments for 96 h and was assessed by counting remaining nuclei. Experiments were performed in three biological replicates.

where few frequently occurring variants have been reported [21]. We identified a total of four OC cases harbouring *RAD51C* c.705G>T suggesting that these individuals might also share common ancestors in FCs. Indeed, another OC case harbouring this *RAD51C* variant was identified in a woman diagnosed with a HGSC of unknown origin (likely upper genital tract) in a hereditary cancer clinic by medical genetic panel testing and was provided to us at the conclusion of this study (**Figure S3.8.**).

The differing carrier frequencies of our variants likely reflect genetic drift due to the waves of localized expansion of the FC population that occurred in Quebec since the founding of this European population in 1608 [21,27]. This change in genetic architecture of the FC population over time has been proposed to account for the varying frequencies of carriers of different pathogenic variants in *BRCA1* and *BRCA2* in this population [20–22]. We cannot exclude the possibility that those that harbour the same variant are closely related as familial associations were not available nor known for all OC cases investigated in this study. We have not determined identity by descent of the more frequently occurring variants as for *RAD51D* c.620C>T [28], due to paucity of cases harbouring such variants in our investigation. It is plausible given the history of the FC population in Quebec that individuals harbouring these variants share common ancestors as we have shown in our studies of the most frequently occurring pathogenic variants in *BRCA1*, *BRCA2*, and *PALB2* [21,23–26,63,68,69].

All variants except *RAD51C* c.705G>T have been reported in OC cases from other populations in the published literature. Our literature review of OC or BC cases with pedigrees (**Figure S3.9.**) showed that those harbouring our candidate variants had a family history of OC (**Figure S3.10.**). It has been shown that pathogenic *BRCA1* or *BRCA2* variant-negative families with a family history of at least two OC cases are more likely to harbour a pathogenic variant in *RAD51C* or *RAD51D* though the overall carrier frequency is lower than that found for pathogenic *BRCA1* or *BRCA2* variant-carriers [5–7,17,70]. Our findings showed that the average ages at diagnosis of all cases harbouring *RAD51C* and *RAD51D* variants are 58.0 and 58.6 years, respectively, which is comparable with a recent population-based study [71]. These observations are consistent with age at diagnosis of cases harbouring pathogenic variants as reported in the NCCN guidelines [72]. However, our study showed that 21% (7/34) of women

harbouring our candidate variants developed OC before the age of 50 years, where the youngest was diagnosed at age of 42. We also showed a higher frequency of the sporadic early-onset OC cases harbouring *RAD51D* variants which is consistent with a previous report [73]. Although we are not able to estimate risk, our data suggests that penetrance might vary in those harbouring pathogenic variants in *RAD51C* or *RAD51D*.

Candidate variants were prioritized for genetic analyses in our study groups based on results from high performance in silico tools for missense variants [41,43]. RAD51D p.Arg232Ter is predicted to affect RAD51D protein production due to premature amino acid termination eliciting nonsense-mediated mRNA decay, rendering this LoF variant compatible with conferring risk for OC [70,74,75] and its classification as pathogenic. The aberrant function of the RAD51C p.Leu138Phe and RAD51D p.Ser207Leu protein variants have been reported independently, where there was a significant reduction of RAD51 foci affecting HR function in the complemented cell lines [5,28]. RAD51D p.Ser207Leu has been shown to disrupt the direct interaction of RAD51D and XRCC2 in RAD51B-RAD51C-RAD51D-XRCC2 (BCDX2) complex reducing HR function [28,76] rendering cells sensitive to PARP inhibitor, olaparib [28] in RAD51D KO cell lines [6]. Our investigation clearly demonstrates that cancer cell lines, including an epithelial ovarian adenocarcinoma cell line characteristic of HGSC OC disease, complemented with the RAD51D p.Ser46Cys have impaired protein expression. Two of the cell lines tested are also sensitive to olaparib (see **Figures 3.4.** and **S3.4.**). Sensitivity may be explained by the weak expression of the protein variant which would impact HR function as was observed by the reduced RAD51 foci formation and increased in γH2AX foci. However, further assays are required to elucidate the underlying mechanism of HR deficiency that resulted in olaparib and talazoparib sensitivity. *RAD51C* c.705G>T; p.Lys235Asn is an interesting missense variant as the nucleotide alteration occurs at the 5′ splice-donor site of the coding region which is predicted to affect splicing by skipping exon four, as was demonstrated by our assays of cDNA from carrier-derived LCLs. We were unable to confirm the presence of exon 4 in the cDNA from non-carrier derived LCLs as cDNA was no longer available. However, our results are consistent with a recent report, which was published during the course of this study, showing that the entirety of exon four of *RAD51C* was excluded from the transcript using a splicing

reporter minigene system of this variant [77]. Interestingly, this report showed compelling evidence that only the transcript lacking exon 4 was only transcribed. Although we cannot exclude the possibility that the exon skipping occurs 100% of the time in cell line model systems applied to assay transcription of this variant, it is difficult to demonstrate that exon skipping also occurs in progenitor cells biologically relevant to the development of OC in carriers. Future studies investigating such variants in cancer predisposition models might be helpful. Notable is that exon 4 of RAD51C encodes the Walker-B ATPase motif (see **Figure 3.3-C**) that is critical for RAD51C function in the HR pathway [78]. Although the mechanisms of aberrant *RAD51C* and *RAD51D* in conferring risk to OC is unknown, our LOH analyses of tumour DNA from carriers are consistent with independent studies that have demonstrated loss of the protein function in tumour cells. Moreover, our LOH analyses of one of the *RAD51D* c.620C>T carriers with bilateral disease suggests that the loss of wild-type *RAD51D* allele was an early event in ovarian tumourigenesis. Collectively, our findings support the application of our bioinformatic pipeline of WES data and selected predictive tools to identify candidate missense variants in *RAD51C* and *RAD51D* suitable for functional validation.

3.7. Conclusion

We were able to identify *RAD51C* and *RAD51D* candidate variants implicated in familial and sporadic OC using our strategy of investigating the germline DNA of the genetically unique FC population that may also be relevant to non-FC populations. Our filtering and prioritizing criteria allowed us to focus on the role of missense variants as candidate OC risk alleles, variants that are more difficult to assess using genetic strategies due to inferences in their role in abrogating gene function. To the best of our knowledge, this is the first report describing *RAD51C* c.705G>T; p.Lys235Asn in the context of hereditary OC, and purporting the clinical relevance of *RAD51D* p.Ser46Cys by our in cellulo assays including olaparib sensitivity. Collectively, our findings suggest that our variants are all likely pathogenic, further supporting the role of *RAD51C* and *RAD51D* in conferring risk to OC.

3.8. Supplementary Materials
The following are available online
at https://www.mdpi.com/article/10.3390/cancers14092251/s1 (also see **Appendix III**).

3.9. Data availability
WES data for familial and sporadic OC cases, CARTaGENE, MNI and Gen3G will be returned to their respective biobanks at the conclusion of our study of OC predisposing genes which is still ongoing. For more information concerning these data contact Patricia N. Tonin at patricia.tonin@mcgill.ca. The data from the analyses of investigation of OCAC, FLOSSIES and gnomAD are available from each of these data resource banks.

3.10. Informed consent statement
Patient consent was waived for this study as study subjects were not directly recruited for this study. The study subjects investigated in this study had been recruited with informed consent to participate in independently established biobanks for research purposes in accordance with ethical guidelines of their respective Institutions Research Ethics Boards. Access to biobanked materials and data are in accordance with their distribution upon request from their respective Institutions Research Ethics Boards and approval for use from this project from The McGill University Health Centre Research Ethics Board.

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3.12. Authors contributions
Conceptualization, W.M.A. and P.N.T.; methodology, W.M.A., L.M., T.R., K.K.O., W.D.F., C.M.T.G., J.-Y.M., J.R. and P.N.T.; investigation and formal analyses, W.M.A., L.M., S.B., C.S., C.T.F., S.L.A., P.N., W.D.F., C.M.T.G., J.-Y.M., J.R. and P.N.T.; cases resources: A.-M.M.-M., D.P., W.D.F. and Z.E.H.; control recourses: D.S., G.R., S.G. and L.B.; writing—original draft preparation, W.M.A. and P.N.T.; writing—reviewing and editing, W.M.A., T.R., K.K.O., L.M., C.T.F., C.S., S.L.A., A.-M.M.-M., D.P., W.D.F., Z.E.H., D.S., G.R., S.G., L.B., C.M.T.G., J.R., J.-Y.M. and P.N.T.; and supervision, P.N.T. All authors have read and agreed to the published version of the manuscript.

3.13. Conflict of interest
The authors declare no conflict of interest.

3.14. Ethical approval

This project was conducted and approved according to the guidelines of The McGill University Health Centre Research Ethics Board (MP-37-2019-4783) (see **Appendix IX**).

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CHAPTER IV: Genetic and Molecular Analyses of Candidate *BRIP1/FANCJ* Variants Implicated in Breast and Ovarian Cancers

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See **Appendix IV** for the permission from Dr. Larissa Milano as the first co-author and **Appendix V** for the permission from all the other co-authors to include this manuscript as Chapter IV of this thesis.

4.1. Preface

Concurrent with the discovery of *RAD51C* and *RAD51D* as ovarian cancer predisposing genes, harbouring pathogenic variants in *BRIP1* was also reported to be associated with an increased risk for developing ovarian cancer. Since this initial report and at the time of starting my Ph.D. program, there were no reports of risk variants in *BRIP1* in ovarian cancer families and cases from the French Canadian population of Quebec.

As part of Chapter III, I demonstrated the spectrum and prevalence of pathogenic variants in *RAD51C* and *RAD51D* in ovarian cancer families and early-onset cases of French Canadian ancestry. During my whole exome sequencing and bioinformatics analyses of these families and cases, no pathogenic variants were identified in *BRIP1*. Therefore, in this chapter, I modified my strategy by investigating rare *BRIP1* variants initially reported in a medical genetic setting at adult hereditary cancer clinics in ovarian and breast cancer cases of French Canadian of Quebec ancestry in order to determine the spectrum and prevalence of these candidate risk *BRIP1* variants in cancer cases and controls of French Canadian ancestry.

4.2. Abstract

Five rare variants in *BRIP1/FANCJ*, initially reported in ovarian (OC) or breast (BC) cancer cases by the adult hereditary cancer clinics, were investigated for their candidacy as clinically relevant variants. These variants were investigated genetically in a population exhibiting genetic drift and molecularly assayed for biological impact. Using in silico tools, population-based genetic databases and other resources, three of the five reported *BRIP1* variants were likely to be damaging: c.797C>T; p.Thr266Met, c.2087C>T; p.Pro696Leu and c.2990_2993delCAAA; p.Thr997ArgfsTer61. The carrier frequencies ranged from 0-0.7% in ancestry defined cancer groups comprised of 47 OC families, 49 hereditary breast and ovarian cancer, 142 hereditary breast cancer syndrome families, 435 sporadic OC cases and 563 sporadic BC cases and 0-0.2% in 1025 population-matched controls. Multiple carriers of the same variants were identified in additional cancer cases. Of the five reported *BRIP1* variants, p.Thr266Met, p.Pro696Leu and p.Thr997ArgfsTer61, which were predicted to be damaging, conferred cellular sensitivity to mitomycin C and cisplatin unlike p.Ser139Ala and p.Ala406Ser. Collectively, our investigation implicates *BRIP1* c.797C>T; p.Thr266Met, c.2087C>T; p.Pro696Leu and c.2990_2993delCAAA; p.Thr997ArgfsTer61 as deleterious variants in OC and BC.

Keywords: *BRIP1*; *FANCJ*; *BACH1*; ovarian and breast cancer; cancer predisposing gene; French Canadian; Genetic drift; mitomycin C sensitivity; cisplatin sensitivity.

4.3. Introduction

BRIP1 has been implicated as a hereditary breast cancer (BC) [233] and ovarian cancer (OC) predisposing gene [234]. *BRIP1* was first reported as a BC predisposing gene in 2006 using a candidate gene approach involving hereditary BC (HBC) syndrome families that were not explained by germline pathogenic variants (PV) in *BRCA1* and *BRCA2* [233]. Subsequent independent studies revealed no association of PVs in *BRIP1* with BC though its role in BC risk remains equivocal [280,283,284,298,384–390]. *BRIP1* was proposed as an OC predisposing gene in 2011 by a genome-wide association study of different cancer cases and controls, including OC [234]. Subsequent studies consistently supported the association of *BRIP1* PVs with OC,

suggesting that such variants in *BRIP1* play a role in conferring increased risk to OC [241]. Carriers of *BRIP1* PVs in OC and BC cases are rare [100]. Fewer than 1-5% of familial and sporadic OC or BC cases harbour PVs in *BRIP1,* which is significantly lower than the 20-80% of familial or 5-20% of sporadic OC or BC carriers of *BRCA1* and *BRCA2* PVs, depending on the population studied [100,240]. While OC and BC cases harbouring *BRIP1* PVs are heterozygous [151], individuals homozygous or compound heterozygous for such variants are associated with the Fanconi anemia (FA) complementation group J (*FANCJ)*, a hereditary bone marrow failure syndrome exhibiting susceptibility to cancer [391–395].

BRIP1, also known as *BACH1* (BRCA1-Associated C-Terminal Helicase), was discovered in the context of elucidating the biological function of BRCA1 [396]. BRIP1 and BRCA1 bind via their BRCA1 Carboxy-Terminus (BRCT) domains: one BRCT motif in BRIP1 and two in BRCA1 [396,397]. The BRCT domain in BRIP1 plays a critical role in its interaction with BRCA1 as a complex along with other proteins in mediating double-stranded DNA break repair by the FA and homologous recombination (HR) pathways [151,391,398,399]. PVs in the BRCT domains of BRIP1 were shown to impact the repair of DNA double-strand breaks due to the loss of interaction between BRIP1 and BRCA1 [202,203,396]. BRIP1 has also seven highly conserved DNA helicase motifs that it is essential for its catalytic activity in processing the repair of DNA inter- or intra-strand crosslinks (ICLs) via the FA-HR pathway [391,396,398–401]. PVs in these helicase motifs have been shown to impact the repair of ICLs due to the loss of BRIP1 catalytic activity [202,203,400,402,403].

In this study, we investigated *BRIP1* variants for their candidacy as clinically relevant variants that were initially reported in BC and OC cases from adult hereditary cancer clinics in the province of Quebec, Canada. We applied a strategy involving the investigation of French Canadians (FC), a population known to exhibit unique genetic architecture due to genetic drift [182,319,320]. The genetic analyses of this population has facilitated the characterization of PVs in known or candidate OC and BC predisposing genes [182]. A small number of PVs in *BRCA1* and *BRCA2* [344,347], one in *PALB2* [366] , *RAD51C* [404] and *RAD51D* [342,404] have been shown to have a higher allele frequency in FC OC and/or BC cases compared with population-matched

controls. *BRIP1* was reported as a cancer predisposing gene based on the investigation of the germline variants in the Icelandic population, a well-documented founder population [405], where *BRIP1* c.2040_2041insTT was reported in 318 OC cases (2.36%) versus 0.41% of population-matched controls [234]. *BRIP1*, however, has not yet been fully investigated in the FC population with only one early study reporting no clinically relevant variants in HBC and hereditary breast and OC (HBOC) syndrome families [406]. We investigated candidate *BRIP1* missense variants using in silico tools selected for their best performance to predict their impact on gene function using a strategy recently applied to investigate missense variants in *RAD51C* and *RAD51D* identified in familial FC OC cancer cases [342,404]. We, then, investigated the carrier frequency of our candidates in genetically defined FC OC and BC cancer and control study groups. We relate our findings to genetically characterized germline *BRIP1* variants identified in the Pan-Cancer OC and BC cases from The Cancer Genome Atlas (TCGA) [407] and cancer-free controls from the Genome Aggregation Database (gnomAD) [408]. As our candidate *BRIP1* variants have not been characterized for their biological impact, we assayed in cell lines complemented with our BRIP1 variants and wild-type (WT) for cellular sensitivity to mitomycin C (MMC), cisplatin and poly (ADP-ribose) polymerase (PARP) inhibitors. Our genetic and molecular investigation of *BRIP1* candidate variants identified in a clinical context of the FC population facilitated the interpretation of candidate variants that are also relevant in other populations.

4.4. Methods

4.4.1. Study groups

The study groups investigated in this report are described in **Table S4.1**. Information concerning *BRIP1* variants in OC or BC cases reported in clinical settings were obtained from adult hereditary cancer clinics in the province of Quebec, Canada. Study groups investigated for *BRIP1* variants were from participants selected from the following established biobanks: Banque de tissus et données of the Réseau de recherche sur le cancer of the Fond de recherche du Québec – Santé (RRCancer biobank) (rrcancer.ca); CARTaGENE (cartagene.qc.ca) [404,409]; Université de Sherbrooke-The Genetics of Glucose regulation in Gestation and Growth (Gen3G) [404,410]; McGill University-

Montreal Neurological Institute (MNI) [404,411]; and The Pan-Cancer – The Cancer Genome Atlas (TCGA) [407]. Clinical data (age of diagnosis, histopathology of cancer, disease stage, and tumour grade), genetic reports, and family history of cancer from selected cases were obtained from the respective biobanks and from adult hereditary cancer clinics. Information for each case was anonymized at source. For further protection of anonymity of participants in this study, we assigned a unique identifier (PT with four digits) to each case and further modified their respective pedigrees. Criteria for denoting FC ancestry is summarized in **Table S4.1**.

This project was conducted with approval and in accordance with the guidelines of The McGill University Health Centre Research Ethics Board (MP-37-2019-4783).

4.4.2. Bioinformatic analyses of BRIP1 variants reported for OC or BC cases of FC ancestry from hereditary cancer clinics

BRIP1 variants initially reported in FC OC or BC cases were provided by adult hereditary cancer clinics (**Table S4.1.**). These cases were also reported negative for PVs in *BRCA1* and *BRCA2*. These reported *BRIP1* variants were re-annotated using the canonical transcript NM_032043.3 [412]. Variants retained for further analyses were those with a minor allele frequency (MAF) ≤0.01 in the general population in the Genome Aggregation Database (gnomAD) v2.1.1. [408,413] and being loss-of-function (LoF) or missense variants classified as PV, likely pathogenic variant (LPV) or variant of uncertain significance (VUS) in ClinVar [414,415] and/or by the American College of Medical Genetics and Genomics (ACMG) guidelines [416]. Missense variants retained for further investigation were those predicted to be conserved and damaging at the RNA or protein level by at least one of the selected in silico tools as described previously [182,289,384,404,417]. These in silico tools were selected based on their best performance [418–421]. Three in silico tools for conservation prediction were applied: Genomic Evolutionary Rate Profiling (GERP++) v1.0 [422]; Phylogenetic P value v4.2 of 100 vertebrates (PhyloP 100 way) [423] and PHAST Conservation v4.2 of 100 vertebrates (PhastCons 100 way) [424] with prediction scores of ≥2.0, ≥0.2 and ≥0.4, respectively. Five in silico tools predicting effect on splicing of transcript were applied: AdaBoost (ADA) v1.1 or Random Forest (RF) v1.1 with prediction scores of ≥0.4 [419],

Maximum Entropy Estimates of Splice Junction (MaxEntScan) v2.0 [425], Human Splicing Finder (HSF) v3.1 [426] and Splice AI [427]. Eight tools predicting effect on protein function were also applied: Rare Exome Variant Ensemble Learner (REVEL) v4.2 [428], Meta-analytic Logistic Regression (MetaLR) v4.2 [429], MetaRNN v4.2. [430], Variant Effect Scoring Test (VEST) v4.2 [431] with prediction scores of ≥0.4, Meta-analytic Support Vector Machine (MetaSVM) v4.2 [429], Eigen v4.2 [432] with prediction scores of ≥0.0, Combined Annotation Dependent Depletion (CADD) v1.6 [433] with prediction score of ≥20 and Protein Variation Effect Analyzer (PROVEAN) v4.2 [434] with prediction score of ≥-2.5.

4.4.3. Determination of frequencies of carriers of BRIP1 variants in defined FC cancer and control study groups

The carrier frequencies of our candidate *BRIP1* variants were investigated in FC study groups that have been extensively characterized in previous reports (**Table S4.1.**). Briefly, *BRIP1* candidate variants were genotyped in peripheral blood lymphocytes (PBL) DNA from index cancer cases from five different FC groups: 47 OC, 49 HBOC and 142 HBC families [344,347,384,404,435] as well as 435 sporadic OC and 563 sporadic BC cases [345,381,384,404], regardless of their status of PVs in *BRCA1* and *BRCA2* (**Table S4.1.**). We genotyped samples using customized TaqMan® [436], Sequenom iPLEX® Gold [437] or Fluidigm® SNP Type™ [438] genotyping assays (primers available upon request) as described previously [381,384,404]. Tumour DNA samples were genotyped where PBL DNA was not available. Carrier frequencies of *BRIP1* candidate variants were determined in population-matched controls by surveying 1025 sequencing-based data from: 433 from Gen3G, 422 from MNI and 170 from CARTaGENE; and surveying 8493 single nucleotide polymorphism (SNP) genotyping-based from CARTaGENE [342,384,404,410,439,440]. For probes of variants not presented on the SNP arrays, pre-phasing and imputation were performed using Eagle2 with the Burrows-Wheeler transformation [441] through Sanger Imputation Services (sanger.ac.uk/tool/sanger-imputation-service/) using Haplotype Reference Consortium release (HRC.r1) v1.1 as a reference [442] as described previously [384,404]. Pair-wise comparisons were performed of carrier frequencies of candidate variants in the different

FC cancer groups versus sequencing-based controls. Two-tailed Fisher's exact test was used to compare carrier frequencies in the cancer versus control groups where un-adjusted *P* values <0.05 for multiple testing was considered significant.

Additional carriers of candidate variants were identified in OC cases from two resources as described in **Table S4.1.**: (1) whole exome sequencing (WES) data was available from 52 sporadic early-onset cancer cases diagnosed with high-grade serous ovarian carcinoma (HGSC) before the age of 50 years [404]; and (2) targeted genotyping of PBL DNA or tumour DNA as described above from 534 recently recruited OC cases [377,404].

Candidate variants were verified in PBL DNA from the identified carriers by bidirectional Sanger sequencing using customized primers (primers available upon request) at the McGill Genome Center as previously described [384,404]. Sequencing chromatograms were visually inspected for variant heterozygosity using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

4.4.4. Determination of carrier frequencies of candidate BRIP1 variants identified in BC and OC cases and controls not selected for FC ancestry

We investigated *BRIP1* candidate variants in genetic data from OC and BC cases from the Pan-Cancer – TCGA [407] and non-cancer controls from the gnomAD v2.1.1. [408,413], and both study groups were not selected for FC ancestry. Variant Call Format (VCF) files that were generated from WES data from the germline of 416 OC and 1072 BC Pan-Cancer – TCGA cases were downloaded as previously described [384,407]. Comma Separated Values (CSV) files that were generated from WES data from the germline of 134,187 cancer-free, non-Finnish European gnomAD v2.1.1. controls were directly downloaded from gnomad.broadinstitute.org. All variants in *BRIP1* were extracted from these files and annotated as previously described [182,289,384]. These variants were subjected to our filtering and prioritizing criteria as described previously [404]. Variants with MAF >0.01 in the general population in gnomAD v2.1.1. were filtered out, and the remaining variants were prioritized for being: (1) LoF or missense variants predicted to affect splicing by at least 1 out of the four in silico tools as described above;

(2) classified as PV or LPVs in ClinVar and/or by ACMG guidelines; (3) predicted to be conserved by at least one of the three selected in silico tools as described above; (4) predicted to be damaging at the level of the protein by at least six of the eight selected in silico tools as described above.

4.4.5. Generation of constructs and cell lines for in cellulo assays of BRIP1 variants

The pcDNA3-3xFlag-*BRIP1*-WT plasmid, expressing the Human BRIP1 Flag tagged with C-terminal 3X DDK tag, was kindly donated by Bob Brosh (NIA/NIH). The pcDNA3-3xFlag-*BRIP1* constructs harbouring one of our *BRIP1* variants were generated via site-directed mutagenesis using Q5® Site-Directed Mutagenesis Kit (New England Biolabs, Canada) with primers listed in **Table S4.2**. The AAVS1 *BRIP1* WT or variant constructs were generated by amplification using the pcDNA3-3xFlag-*BRIP1* plasmids and primers listed in **Table S4.2**. Products were cloned into the AAVS1 vector in NotI/PspXI sites [443].

The U2OS (sarcoma derived cell line) and Hela (cervical carcinoma derived cell line) BRIP1 knock-out (KO) and control cells were kindly donated by Sharon Cantor [444,445] and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin. BRIP1 KO cells were stably complemented using the AAVS1 genomic editing system [443]. Briefly, cells were transfected with 4 µg of the AAVS1 construct containing either the WT or one of the BRIP1 variants, along with the 0.4 µg of the pZFN plasmid for 4h using Lipofectamine 2000 (Invitrogen, Canada). After 24h, transfected cells were selected with Gibco™ Geneticin™ Selective Antibiotic (G418 Sulfate) for 7 days. Established cell lines containing the BRIP1 variants were maintained in DMEM supplemented with 10% FBS, 1% Penicillin-Streptomycin and 0.5 mg/ml of G418 Sulfate.

4.4.6. Drug sensitivity assays

The U2OS or Hela cells were seeded in triplicate assays into a Corning 3603 black-sided clear bottom 96-well microplate at a density of 2000 cells per well. MMC, cisplatin and PARP inhibitors (olaparib and talazoparib) sensitivity assays were, then, performed as previously described [446]. Cells were treated with the indicated drugs for 4 days with

concentrations ranging from 0 to 8 ng/ml for MMC, 0 to 60 µM for cisplatin, 0 to 2.5 µM for olaparib and 0 to 40 nM for talazoparib. The entirety of each well was imaged at 4x with Cytation 5 Cell Imaging Multi-Mode Reader and the Hoechst-stained nuclei were quantified using the Gen5 Data Analysis Software v3.03 (BioTek Instruments). Cell viability was expressed as percentage of survival of treated cells relative to vehicle-treated cells. Results represent the mean ± standard error of the mean (SEM) of at least 3 independent experiments, each performed in triplicate.

4.4.7. Protein extraction and immunoblotting assays

Total soluble protein extracts and immunoblotting were performed as previously described [447]. BRIP1 protein expression was detected using a polyclonal antibody (Sigma, #B1310). Anti-Tubulin (Abcam, #ab7291) served as the loading control. Anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch) conjugated to horseradish peroxidase were used as secondary antibodies.

4.5. Results

4.5.1. Candidate BRIP1 variants were reported in FC probands with OC and BC by adult hereditary cancer clinics

We received information from three OC and four BC probands who tested positive for a *BRIP1* variant based on a 23-to-34 gene-panel testing for germline variants and copy number variants (CNV), except for two cases where CNV testing was done for only *BRCA1* and *BRCA2.* All seven probands self-reported FC of Quebec ancestry. Medical genetic reports revealed that the four BC probands from independent families harboured a frameshift c.2990_2993delCAAA; p.Thr997ArgfsTer61 or missense variant c.415T>G; p.Ser139Ala in *BRIP1*, and the three OC probands each harboured a missense variant c.797C>T; p.Thr266Met, c.1216G>T; p.Ala406Ser or c.2087C>T; p.Pro696Leu in the same gene as shown in **Figure S4.1**. These probands were negative for PVs, LPVs or VUS variants and CNVs for all genes tested by the panels, including in *BRCA1* and *BRCA2.*

All five *BRIP1* variants were re-annotated for further characterization as candidates for the study. All missense variants were predicted to be at conserved amino acid

residues by all three selected in silico tools and were also predicted to be damaging at the protein level by at least one of the eight selected tools (**Table 4.1.**). The p.Thr266Met and p. Pro696Leu were predicted to be damaging by all eight in silico tools, whereas p.Ala406Ser and p.Ser139Ala were predicted to be damaging by one or three out of the eight tools, respectively. None of the missense variants were predicted to affect splicing of *BRIP1* transcript by any of the tools.

As described in **Table 4.1.**, c.2990_2993delCAAA; p.Thr997ArgfsTer61, which was reported in two probands diagnosed with BC, is rare in the cancer-free, non-Finnish European population, having a MAF of 6.8e-5, and in other populations (**Table S4.3.**). It has been classified as PV/LPV in ClinVar (Accession number: VCV000234281.23) and PV by ACMG guidelines (Pathogenic Very Strong level 1 [PVS1]; Pathogenic Supporting level 5 [PP5]; and Pathogenic Moderate level 2 [PM2]). This is under the assumption that the encoded protein has a disrupted BRCT domain that would affect its interaction with BRCA1 based on a previous report that demonstrated such impact by another frameshift variant in *BRIP1* c.2992_2995del; p.Lys998GlufsTer60 [448], which is located adjacent to our variant. Our *BRIP1* frameshift variant is predicted to introduce a premature termination codon at amino acid position 61 and induce truncation of the encoding protein in the BRCT domain, if synthesized (**Figure 4.1-A**). There have been multiple reports in ClinVar of c.2990_2993delCAAA; p.Thr997ArgfsTer61 in the context of hereditary OC, BC as well as FA. The family history of this *BRIP1* frameshift variant carrier proband (PT0152) from family F1646 was consistent with features of HBC syndrome, having an early age of onset of BC (diagnosed at 34 years) and other relatives with an early age of diagnosis with BC (**Figure S4.1.**). Whereas the same frameshift variant carrier BC proband (PT0164) from family F1656 (diagnosed at 35 years) was less indicative of harbouring features of a known hereditary cancer syndrome phenotype, though multiple types of cancer were reported in this family.

The four *BRIP1* missense variants of interest were all rare in different populations (**Table S4.3.**), with MAF of at least 1.0e-4 in the non-Finnish European controls, or have not been identified as with c.1216G>T; p.Ala406Ser. Two probands PT0147 from family F1641 and PT0149 from family F1642 were both diagnosed with BC at ages 48 and 37 years, respectively, and were reported to harbour c.415T>G; p.Ser139Ala (**Figure**

Table 4.1. Characteristics of *BRIP1* candidate variants identified in carrier probands with ovarian or breast cancer cases of French Canadians from the adult hereditary cancer clinics.

Genomic features (hg19/GRCh37)					
Genome change	g.59926582A>C	g.59885949G>A	g.59876585C>A	g.59853772G>A	g.59761413delTTTG
Coding change	c.415T>G	c.797C>T	c.1216G>T	c.2087C>T	c.2990_2993delCAAA
Protein change	p.Ser139Ala	p.Thr266Met	p.Ala406Ser	p.Pro696Leu	p.Thr997ArgfsTer61
Probands					
OC cases (n=3)	0	1	1	1	0
BC cases (n=4)	2	0	0	0	2
Allele frequencies in gnomAD[1]					
Non-Finnish European	8.5e-5 (10/118008)	5.1e-5 (6/117792)	-	1.0e-4 (12/118082)	6.8e-5 (7/102586)
Clinical classification[2]					
ClinVar (number of submissions)	VUS (14)	VUS (7)	VUS (2)	VUS (10)	PV/LPV (14)
ACMG guidelines (implemented rule)	VUS (PM2)	VUS (PM2; PP3)	VUS (PM2)	VUS (PM2; PP3)	PV (PVS1; PP5; PM2)
Predictions by in silico tools[3]					
GERP++ v1.0	Conserved	Conserved	Conserved	Conserved	-
PhyloP 100 way v4.0	Conserved	Conserved	Conserved	Conserved	-
PhastCons 100 way v4.0	Conserved	Conserved	Conserved	Conserved	Conserved
REVEL v4.2	Benign	Pathogenic	Benign	Pathogenic	-
MetaLR v4.2	Tolerated	Damaging	Tolerated	Damaging	-
MetaSVM v4.2	Tolerated	Damaging	Tolerated	Damaging	-
MetaRNN v4.2	Tolerated	Damaging	Tolerated	Damaging	-
CADD v1.6	Damaging	Damaging	Damaging	Damaging	-
VEST v4.2	Damaging	Damaging	Tolerated	Damaging	-

EIGEN PC v4.2	Pathogenic	Pathogenic	Benign	Pathogenic	-
PROVEAN v4.2	Neutral	Damaging	Neutral	Damaging	-

Annotation of candidate variants based on the *BRIP1* transcript NM_032043.3 in the National Center for Biotechnology Information (NCBI) - Reference Sequence (RefSeq) database (tark.ensembl.org/web/manelist/) [412]; [1] Allele frequencies in non-cancer, non-Finnish European controls from the Genome Aggregation Database (gnomAD) v2.1.1 (gnomad.broadinstitute.org) [413]; [2] Clinical classifications from ClinVar (ncbi.nlm.nih.gov/clinvar/) [414,415] based on last revision in April 2022, and the American College of Medical Genetics and Genomics (ACMG) guidelines [416,449]; and [3] Applied predictive in silico tools for conservation and damaging at the protein level selected based on their best performance [418].

BC: Breast cancer; OC: Ovarian cancer; PM2: Pathogenic Moderate level 2; PP3: Pathogenic Supporting level 3; PP5: Pathogenic Supporting level 5; LPV: Likely Pathogenic Variant; PV: Pathogenic Variant; PVS1: Pathogenic Very Strong level 1; VUS: Variant of Uncertain Significance; and (-): Not applicable/reported.

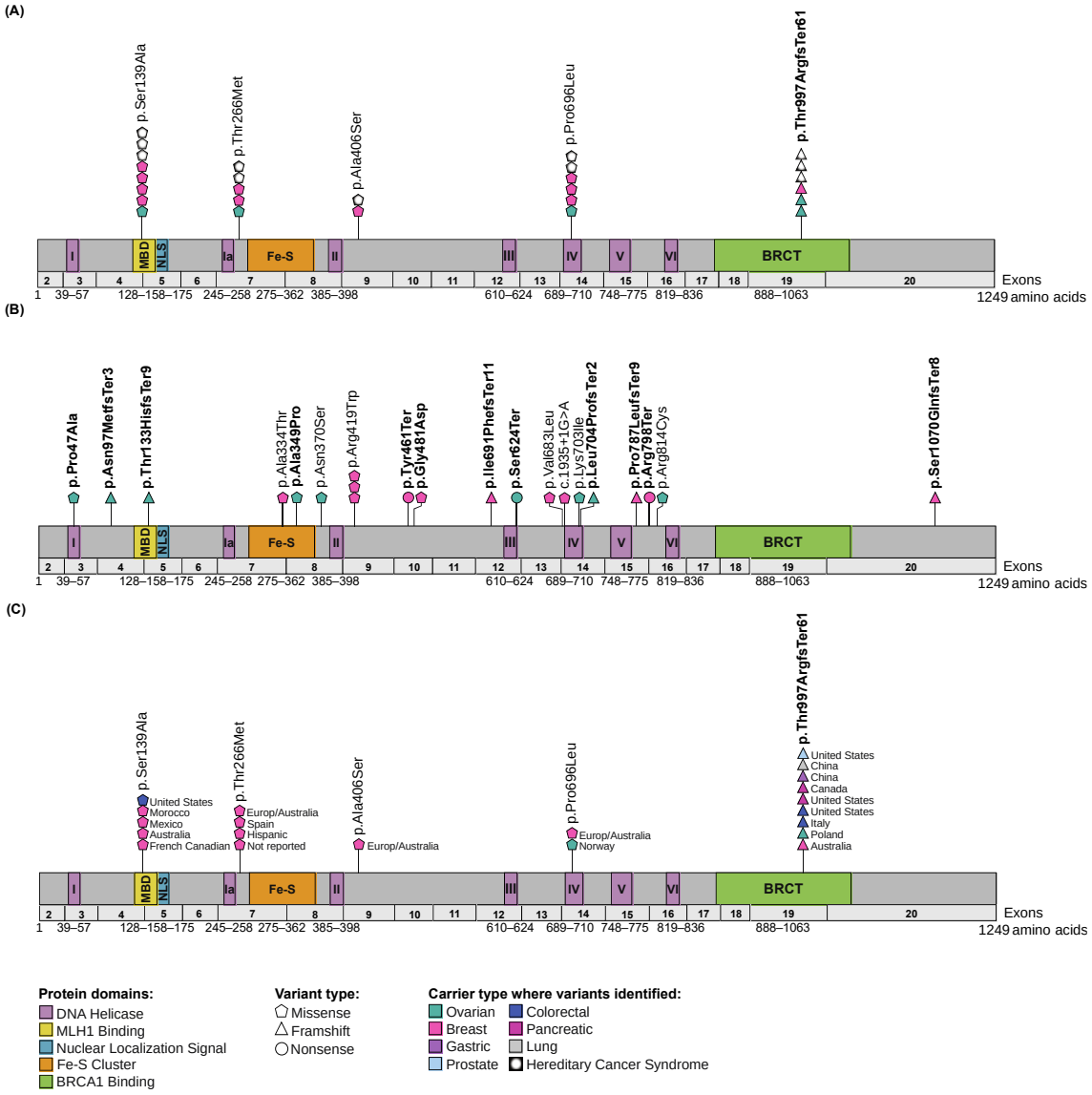

Figure 4.1. The location of *BRIP1* candidate variants in the transcript and protein domains.

Diagram of *BRIP1* transcript with protein domains indicating the location of candidate variants (see **Table S4**) identified in: (A) French Canadian ovarian (OC) and breast (BC) cancer cases in this study; (B) OC and BC Pan-Cancer cases from The Cancer Genome Atlas project, mostly of European origin in this study; and (C) different cancers harbouring one of the candidate variants in FC OC or BC cases reported in the literature review. *BRIP1* candidate variants classified as PV in ClinVar (ncbi.nlm.nih.gov/clinvar/) [414,415] or by the American College of Medical Genetics and Genomics guidelines [416] are bolded. *BRIP1* transcript (NM_032043.3) and protein domains was based on

the National Center for Biotechnology Information - Reference Sequence database (ncbi.nlm.nih.gov/protein/NP_114432.2) and (tark.ensembl.org/web/manelist/) [412].

S4.1.). This variant was classified as VUS in ClinVar (VCV000132712.21) in the context of hereditary OC, BC or FA and by the ACMG Guidelines because of its rarity in the cancer-free controls (Pathogenic Moderate [PM2]). The effect of this amino acid substitution in BRIP1 is unknown though it is located in the MLH1 binding domain (MBD) [450] (**Figure 4.1-A**). Interestingly, proband PT0147 (Family F1641) reported a family history of cancer suggestive of Lynch syndrome [451], while proband PT0149 (Family F1642) reported a family history of multiple types of cancer (**Figure S4.1.**). None of these families had a confirmed case of OC though there was a second degree relative of proband PT0147 (Family F1641) suspected of having either uterine cancer or OC. In contrast to these carriers, the probands harbouring the missense variants c.797C>T; p.Thr266Met (PT0150 from family F1636), c.1216G>T; p.Ala406Ser (PT0151 from family F1645) or c.2087C>T; p.Pro696Leu (PT0099 from family F1628) were diagnosed with OC at ages 54, 62 and 33 years, respectively (**Figure S4.1.**). These missense variants were classified as VUS in the context of hereditary OC, BC or FA in ClinVar (VCV000128196.14; VCV000407821.7; and VCV000128167.23, respectively) and by ACMG guidelines because of their rarity in the cancer-free controls (Pathogenic Moderate Level 2 [PM2]) and in silico predictions (Pathogenic Supporting Level 3 [PP3]) [416]. The effect of these amino acid substitutions in BRIP1 is also unknown though one is located in the DNA helicase domain (**Figure 4.1-A**). Though the family history of proband PT0151 (F1645) is suspicious for Lynch syndrome, with two reports of intestinal or colon cancers, the families of all the OC probands harbouring these missense variants reported multiple cancer types. There were no striking characteristics of the family history of cancer in probands PT0150 (Family F1636) and PT0099 (Family F1628) (**Figure S4.1**).

4.5.2. Multiple carriers of candidate BRIP1 variants were identified in defined FC cancer study groups

The carrier frequencies of *BRIP1* candidate variants were determined in FC study groups comprised of familial and sporadic OC or BC cases, regardless of their status of *BRCA1* and *BRCA2* PVs and in population-matched controls. We did not identify any other carriers of c.2990_2993delCAAA in any of our study groups. However, we identified carriers of each of our missense variants in at least one of our FC study groups (**Table 4.2.**). We determined that the frequency of cancer carriers ranged from 0-0.7% versus 0-0.2% in the population-matched controls, depending on the variant and the group investigated (**Table 4.2.**). However, the carrier frequency of each variant in a defined cancer group was not significantly different to that of the control group. It is interesting to observe that carriers of missense variants were mostly identified in sporadic BC cases in contrast to OC cases.

Table 4.2. Carrier frequencies of candidate *BRIP1* variants in French Canadian cancer cases and population-matched controls.

***BRIP1* variant**	**Study groups[1]**	**Cancer cases tested[1]**	**Number of tested participants (or families) per study group**	**Number of variant carriers (%)**	***p*-value[2]**
c.415T>G; p.Ser139Ala	OC families	OC	66 (47)	0	-
	HBOC families	OC or BC	49 (49)	0	-
	HBC families	BC	142 (142)	0	-
	Sporadic OC cases	OC	435	0	-
	Sporadic BC cases	BC	563	**1/563 (0.2)**	1.000
	FC sequencing-based controls	-	1025	**2/1025 (0.2)**	-
c.797C>T; p.Thr266Met	OC families	OC	66 (47)	0	-
	HBOC families	OC or BC	49 (49)	0	-
	HBC families	BC	142 (142)	**1/142 (0.7)**	0.122
	Sporadic OC cases	OC	435	**1/435 (0.2)**	0.298
	Sporadic BC cases	BC	563	**2/563 (0.4)**	0.126
	FC sequencing-based controls	-	1025	0	-
c.1216G>T; p.Ala406Ser	OC families	OC	66 (47)	0	-
	HBOC families	OC or BC	49 (49)	0	-

	HBC families	BC	142 (142)	0	-
	Sporadic OC cases	OC	435	0	-
	Sporadic BC cases	BC	563	**2/563 (0.4)**	0.126
	FC sequencing-based controls	-	1025	0	-
c.2087C>T; p.Pro696Leu	OC families	OC	66 (47)	0	-
	HBOC families	OC or BC	49 (49)	0	-
	HBC families	BC	142 (142)	0	-
	Sporadic OC cases	OC	435	0	-
	Sporadic BC cases	BC	563	**1/563 (0.2)**	0.355
	FC sequencing-based controls	-	1025	0	-
c.2990_2993delCAAA; p.Thr997ArgfsTer61	OC families	OC	66 (47)	0	-
	HBOC families	OC or BC	49 (49)	0	-
	HBC families	BC	142 (142)	0	-
	Sporadic OC cases	OC	435	0	-
	Sporadic BC cases	BC	563	0	-
	FC sequencing-based controls	-	1025	0	-

[1] Study groups comprised of ovarian cancer (OC), breast cancer (BC), hereditary breast and ovarian cancer (HBOC) syndrome and hereditary breast cancer (HBC) syndrome families, all from the French Canadian (FC) population of Quebec; and [2]Two-tailed *p*-values (not adjusted for multiple testing) calculated using Fisher's exact test in pair-wise comparisons between variant carriers of each cancer study group and population-matched controls. (-) denotes not applicable.

It was not possible to determine the carrier frequency of any of the *BRIP1* candidate variants in 8493 genotyping-based FC controls (**Table S4.1.**) as none of these variants were represented on any of these genotyping arrays. However, we were able to impute c.797C>T as this variant was present in the HRC.r1 haplotype reference panel; no carriers were identified among the genotyping-based controls, suggesting that this variant is rare in the cancer-free FC population.

With our expectation that some of our candidates may occur with a higher frequency in the FC population due to genetic drift [182], we investigated our candidate

BRIP1 variants in additional cancer cases. We genotyped PBL DNA or surveyed available genetic data of 534 additional OC and 52 sporadic early-onset OC cases for carriers of our candidate *BRIP1* variants. We identified four carriers among the additional group of OC cases, and none in the early-onset cases, harbouring c.415T>G; p.Ser139Ala (PT0120), c.1216G>T; p.Ala406Ser (PT0200), c.2087C>T; p.Pro696Leu (PT0119) or c.2990_2993delCAAA; p.Thr997ArgfsTer6 (PT0156) (**Table S4.4.**). The carrier frequency of each variant in these study groups was less than 2%, which is consistent with the low carrier frequency observed for these variants in the other defined FC study groups (**Table 4.2.**).

4.5.3. Genetic analyses of BC and OC cases and controls not selected for FC ancestry identified candidate BRIP1 variants

Using our criteria for identifying clinically relevant candidate *BRIP1* variants, we investigated genetic data from the germline of 412 OC and 1072 BC Pan-Cancer – TCGA cases and 134,187 cancer-free, non-Finnish European controls**.** We identified carriers of nine variants in 8/412 (1.9%) OC cases and ten variants in 9/1076 (0.8%) BC cases (**Figure 4.1-B** and **Table S4.4.**), which included one BC carrier of c.415T>G; p.Ser139Ala, a candidate variant that was also identified in our FC cases (**Table 4.1.**). These variants were identified in 0.001-0.09% of the non-cancer, non-Finnish European controls in gnomAD (**Table S4.4.**). There were ten LoF (three nonsense, six frameshift and one canonical alternative splicing) and nine missense variants, including c.1109A>G; p.Asn370Ser that was predicted by SpliceAI [427] to affect splicing that may result in donor loss (**Table S4.4.**). Eight of these variants were located in biologically relevant domains of BRIP1 comprised of the MBD and iron-sulfur (Fe-S) [452] and one of the DNA helicase motifs [403] (**Figure 4.1-B**).

4.5.4. In cellulo assays revealed deleterious BRIP1 candidate variants affected cellular sensitivity to cisplatin but not to olaparib or talazoparib

To explore the functionality of BRIP1 protein encoded by candidate variants identified in our FC study groups, we generated stable cell lines expressing: p.Ser139Ala, p.Thr266Met, p.Ala406Ser, p.Pro696Leu, p.Thr997ArgfsTer61, a BRIP1 WT and an

empty vector (EV) using the AAVS1 genomic editing system in BRIP1-depleted cells (**Figure 4.2-A-B**) [443]. Two *BRIP1* variants were included as controls: c.1045G>C; p.Ala349Pro [202,203], which is classified in ClinVar as PV/LPV (VCV000030535.14) or as a VUS by ACMG (Pathogenic Moderate level 2 [PM2]) and c.2220G>T; p.Gln740His [202,203], which is classified in ClinVar as likely benign (VCV000133752.34) or as a VUS by ACMG (Pathogenic Moderate level 2 [PM2]). BRIP1 p.Ala349Pro was selected as a positive control which was predicted to be damaging at the protein level by six out of our eight selected in silico tools [404,417]; and BRIP1 p.Gln740His was selected as a negative control which was predicted to be damaging by only one out of the eight predictive tools.

BRIP1 activity is critical for mediating the repair of DNA ICLs, and cells deficient for this gene are sensitive to ICL-inducing agents such as MMC and cisplatin [450,453]. Given this phenotype, we assessed the sensitivity of the cells containing the interrogated BRIP1 variants to increasing concentrations of either MMC or cisplatin. As expected, Hela BRIP1 KO cells complemented with the EV were more sensitive to both MMC and cisplatin when compared to the Hela control cells (Ctl) (**Figure 4.2-C** and **Figure S4.2.**) [203]. Complementation with the BRIP1 WT rescued the cells sensitivity from the effect of ICL-inducing agents. This phenotype was also observed in BRIP1 KO cells complemented with BRIP1 p.Gln740His, the negative control. However, cells complemented with BRIP1 p.Ala349Pro, the PV/LPV control, failed to confer resistance to MMC or cisplatin (**Figure 4.2-C** and **Figure S4.2.**). A similar profile was observed in cells complemented with the EV. As previously demonstrated [203], greater sensitivity to MMC and cisplatin was also observed in BRIP1-depleted U2OS cells complemented with the EV or BRIP1 p.Ala349Pro, and resistance was partially recovered with the BRIP1 WT (**Figure S4.3.**).

We next determined whether BRIP1-depleted cells expressing the selected BRIP1 variants were able to confer resistance to the ICL-inducing agents MMC and cisplatin (**Figure 4.3.** and **Figure S4.2.**). BRIP1 p.Ser139Ala and p.Ala406Ser behaved similarly to the BRIP1 WT in terms of the ability to rescue the viability of BRIP1-depleted cells to MMC and to cisplatin, indicating that these missense variants do not impact the functionality of BRIP1 to resolve ICLs (**Figure 4.3-A-B**). However, cells expressing

BRIP1 p.Thr266Met, p.Pro696Leu or p.Thr997ArgfsTer61 were unable to rescue the sensitivity of BRIP1-depleted cells. BRIP1 p.Thr266Met and p.Pro696Leu stood out with the highest sensitivity to the ICL-inducing drugs (**Figure 4.3-A-B**), with survival percentages each of 51% relative to the WT at dose of 2ng/ml of MMC and 61% and 60% relative to WT at a dose of 30µM of cisplatin, respectively. Using the same criteria, the relative survival of EV was 46% to MMC and 52% to cisplatin, BRIP1 p.Ala349Pro was 41% to MMC and 56% to cisplatin, while BRIP1 p.Thr997ArgfsTer61 sensitivity was 60% and 73% to MMC and cisplatin, respectively (**Figure 4.3-A-B**).

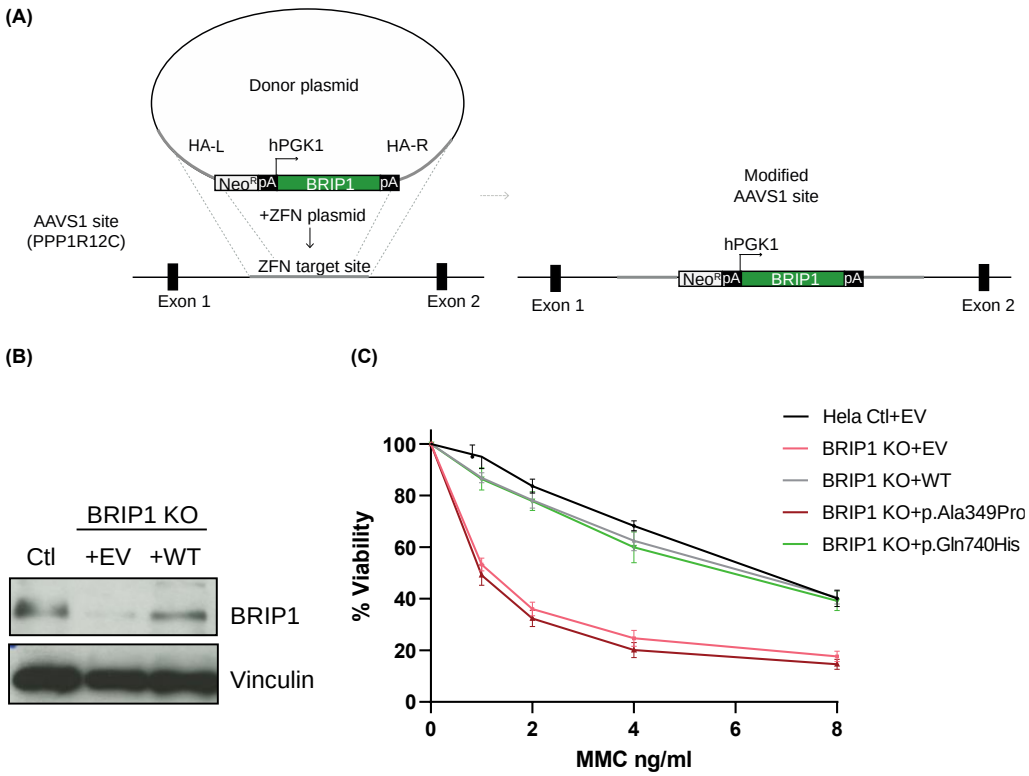

Figure 4.2. Strategy used to assess the functional impact of BRIP1 variants in a mammalian cell system.

(A) Scheme representing the AAVS1 genomic editing system used to complement BRIP1 knock-out (KO) cells. (B) Hela control (Ctl) cells and BRIP1 KO cells completed with empty vector (EV) or BRIP1 wild-type (WT) using the AAVS1 system were confirmed by western blot analyses of BRIP1 protein expression, and vinculin was used as a loading control. (C) Survival curves of Hela BRIP1 cells stably complemented with constructs of BRIP1 WT, BRIP1 p.Ala349Pro (a PV/LPV as a control), BRIP1

p.Gln740His (a likely benign variant as a control), or EV and plated in triplicate in a 96 well plate. Cell viability was monitored following MMC treatment for 96 hours and was assessed by counting remaining nuclei. Experiments were performed in three biological replicates.

These results provide evidence in favor of the possible impaired function of BRIP1 p.Thr266Met, p.Pro696Leu and p.Thr997ArgfsTer61 variants. Survival curves for all variants are depicted in **Figure S4.2**. For further validation, variants were also tested in the U2OS BRIP1-depleted cell line (**Figure S4.3.**). Similar results to those obtained in Hela cells were observed. However, the dynamic range between U2OS BRIP1-depleted cells complemented with the EV and the WT was proportionately smaller relative to Hela cells. In U2OS cells, BRIP1 p.Ala406Ser appears to have a partial complementation (**Figure S4.3-C** and **I**). Concerning protein expression, the BRIP1 variants tested here lead to a protein product as detected by immunoblotting in our experimental Hela and U2OS cell line models (**Figure 4.3-E**, **Figure S4.2-K** and **Figure S4.3-K**). Considering the impact of PARP inhibitors on the clinical management of BC and OC patients [454], cells expressing our BRIP1 variants were also tested for the sensitivity to PARP inhibitors, olaparib or talazoparib. As previously demonstrated [455], cells depleted in BRIP1 have no greater sensitivity to either olaparib or talazoparib. Complementation with the BRIP1 WT or any of our BRIP1 variants did not alter the resistance profile to either PARP inhibitor (**Figure 4.3-C-D** and **Figure S4.4.**).

4.6. Discussion

We investigated five rare *BRIP1* variants that were initially reported in OC or BC cases by the adult hereditary cancer clinics for candidacy as clinically relevant variants. Our genetic analyses of these variants: (1) assessed bioinformatically their potential impact on gene function; (2) investigated their carrier frequency in defined cancer study groups comprised of familial and sporadic OC and BC cases and population-matched controls from a population exhibiting genetic drift; and (3) assessed bioinformatically other candidate variants in *BRIP1* and investigated their carrier frequency in ancestrally diverse cancer study groups and controls. We also assayed biologically, the impact of

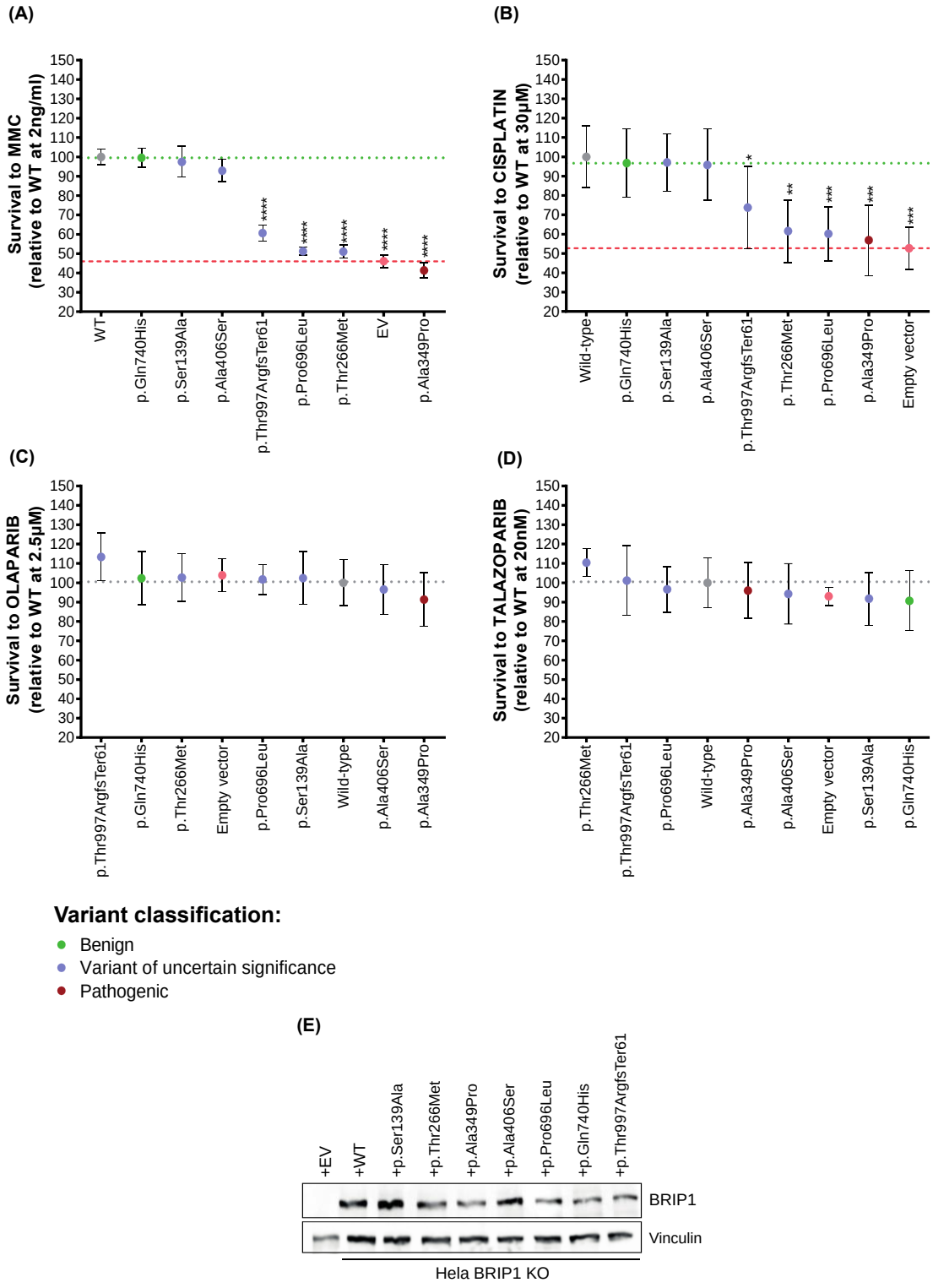

Figure 4.3. Drug sensitivity of cells expressing BRIP1 variants to DNA inter- and intra-strand crosslinks inducing agents and poly-ADP-ribose polymerase inhibitors.

Sensitivity of BRIP1 knock-out (KO) cells stably complemented with constructs of BRIP1 variants or empty vector to the DNA inter- or intra-strand crosslinks (ICLs) inducing agents: (A) Mitomycin C (MMC) and (B) cisplatin; and to poly-ADP-ribose polymerase (PARP) inhibitors (C) olaparib and (D) talazoparib. Sensitivity profiles were determined with the BRIP1 wild-type set as 100% at the concentrations of 2ng/ml for MMC (A), 30μM for cisplatin (B), 2.5μM of olaparib (C) and 20nM for talazoparib (D). Survival data from each BRIP1 variant are sorted in descending order in response to drug sensitivity and presented based on the mean (±standard error of the mean [SEM]) from at least 3 independent experiments, performed in triplicate. Statistical significance was determined by One-way ANOVA test with Dunnett's multiple comparison post-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$ and (****) $P < 0.0001$. (E) Western blot analysis showing BRIP1 protein expression in BRIP1-depleted cells after stable complementation with the indicated variants, with vinculin as a loading control.

these five *BRIP1* variants on the encoded protein function based on the current known role of BRIP1 in DNA repair [151] and in cellulo sensitivity to chemotherapeutic agents such as MMC and cisplatin and targeted therapeutic agents such as PARP inhibitors [7,91]. Collectively, our findings from these assays suggest that a frameshift variant c.2990_2993del; p.Thr997ArgfsTer6 and two of the four missense variants c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu likely affect BRIP1 function.

The identification of multiple carriers of each of our *BRIP1* candidate variants is likely attributable to shared ancestry of the FC population of Quebec [342,347,352]. We could not determine whether there was a shared genome segment identical-by-descent due to the paucity of carriers of each variant though we have been able to demonstrate shared ancestry using haplotype analyses of the carriers of most frequently occurring PVs in *BRCA1*, *BRCA2* [347,352,456], *PALB2* [366], *RAD51C* [404] and *RAD51D* [342,404] in the context of OC and BC in the FC population [182]. In 2008, an early independent study of *BRIP1* in FC BC cases from HBC or HBOC families of Quebec reported 42 variants in *BRIP1* but concluded that none are likely clinically relevant [406]. We reassessed these variants with our selected in silico tools and retrieved current information from genetic databases (see **Table S4.6.**), and indeed, none were predicted

to be biologically relevant. The only variant found in common with our study of the FC cancer cases was c.415T>G; p.Ser139Ala, but we showed that this variant is unlikely to affect the protein function. We classified 86% of these variants as benign or likely benign based on reports in ClinVar and/or by ACMG guidelines, which is not surprising as 50% of variants are common having MAF>0.01 in the FC controls. The genetic heterogeneity observed in *BRIP1* variant carriers is consistent with the germline genetic landscape of the FC population of Quebec [182]. The differences in carrier frequencies of our variants in *BRIP1* as well as those observed in *BRCA1*, *BRCA2, PALB2, RAD51C* and *RAD51D* are expected in FCs and consistent with the genetic drift that has been attributed to the waves of localized expansion of this population that occurred in Quebec since 1608 [322,327,334]. Given the European ancestry of FCs, it is not surprising that all five candidate *BRIP1* variants were also identified in the germline of cancer cases in the literature (see **Figure 4.1-C**). Moreover, the overall low carrier frequency of candidate *BRIP1* variants in FC cancer study groups and the Pan-Cancer – TCGA cases is consistent with the overall low carrier frequency (approximately <2%) of *BRIP1* PVs that have been reported in cancer cases from other populations [100,280].

Although the role of our candidate *BRIP1* variants in conferring risk to OC and BC remains to be determined, our in cellulo analyses suggest that some affect BRIP1 function. BRIP1 binds directly to BRCA1 via BRCT motifs which play a critical role in BRCA1 stability to mediate repair of double-stranded DNA breaks [151,187,241]. Based on our current knowledge, PVs in the BRCT domain of BRIP1 negatively affected the repair of double-stranded DNA breaks by abrogating the BRIP1-BRCA1 interaction [396] rendering cells sensitive to cisplatin [202,203,403]. We showed in our in cellulo assays, that our BRIP1 variant p.Thr997ArgfsTer61 predicted to affect the BRCT domain, though expressed impaired cellular sensitivity to MMC and cisplatin. Though speculative, the ability of BRIP1 p.Thr997ArgfsTer61 to interact with BRCA1 may have been impaired as a consequence of the loss of an intact BRCT domain for BRCA1 binding. Another frameshift variant in *BRIP1* c.2992_2995del; p.Lys998GlufsTer60 which affects an adjacent amino acid has also been shown to be expressed in cells [448], suggesting that transcripts from these variants may not elicit nonsense mediated decay. Our functional assessment determined that candidate variants p.Thr266Met, p.Pro696Leu and

p.Thr997ArgfsTer61 exhibited loss of BRIP1 WT function upon exposure to MMC and cisplatin, while p.Ser139Ala and p.Ala406Ser did not alter cellular sensitivity to these ICL-inducing agents. The proximity of p.Thr266Met and p.Pro696Leu to any one of the helicase domains in BRIP1 may account for effect on the protein function [202,203] in our assays and warrants further biochemical characterization of helicase activity. Though lack of BRIP1 results in HR deficiency and loss of replication fork protection, it does not result in PARP inhibitor-induced single-stranded DNA breaks [455]. Thus, none of the five variants expressing cells exhibited sensitivity to PARP inhibitors, consistent with independent reports of response to WT and variant BRIP1 [455,457]. This may have clinical implications for the management of OC and BC patients who are carriers of *BRIP1* PVs [99,286] and (nccn.org/guidelines/category_2). Indeed, it has been shown that BC tumour DNA from *BRIP1* carriers did not exhibit a mutational signature characteristic of HR defects a signature exhibited in *BRCA1* and *BRCA2* carriers exhibiting sensitivity to PARP inhibitors [458]. Although we were able to cultivate BRIP1-deficient cell lines as also reported by other groups, we had considerable difficulty performing complementation of small interfering RNA (siRNA) BRIP1-deficient cells with a WT construct using a transient transfection system [202]. To overcome this issue, we generated stable cell lines using the AAVS1 system in a CRISPR Cas9 KO background that was able to rescue BRIP1 WT protein. Genomic editing using a donor guide containing the studied variants could be applied to further overcome this barrier.

The bioinformatic tools selected to predict the effect our missense candidate variants on protein function align in part with the results of our MCC and cisplatin sensitivity assays. It is notable that six out of the eight in silico tools, selected for their best performance [418,421], predicted that p.Thr266Met and p.Pro696Leu to be damaging. In contrast, only one in silico tool predicted that p.Ala406Ser and p.Ser139Ala to be damaging, and two other tools also predicted that p.Ala406Ser to be damaging. The prediction scores of the positive and negative controls, p.Ala349Pro and p.Gln740His [202,203], which are classified as LPV and benign, respectively in the ClinVar Database and by ACMG guidelines, were consistent with our expectation of these variants as positive and negative controls in our assays. Our observations

highlight the relevance of performing functional assays on missense variants, when possible, though this may not be feasible in medical genetics settings.

Due to the small number of carriers in cancer cases, particularly in familial cases, this study was underpowered to address differences in *BRIP1* carrier frequencies in OC versus BC cases in our FC population. Moreover, it was not feasible to screen all the FC cancer cases investigated in this study for *BRIP1* variants. Nonetheless, there were more carriers of c.797C>T; p. Thr266Met and c.2087C>T; p.Pro696Leu in sporadic BC cases (5/563, 0.9%) versus sporadic OC cases (1/435, 0.2%) though this was not statistically different. It was not unexpected to find a carrier among our HBC families (1/142; 0.7%), given the fact that *BRIP1* was originally reported as a BC predisposing genes by investigating HBC families [233]. One of the first reports investigating the germline of selected candidate genes involved in the HR pathway in sporadic OC cases reported four carriers of *BRIP1* PVs, two with a family history of BC [251]. A recent population-based study investigating genes involved in BC risk reported a statistical difference in carriers of *BRIP1* LoF variants in cases with a family history of BC versus controls (20/6361 [0.31%]; odds ratio= 2.15; 95% Confidence Interval [CI]: 1.25 to 3.58); p=0.004) [283], a result consistent with another study [280] and the original report describing PVs in *BRIP1* in familial BC cases [233].

A literature review of our candidate *BRIP1* variants, revealed that one of our PV/LPV, c.2990_2993delCAAA; p.Thr997ArgfsTer61, occurred in the context of hereditary cancers other than BC or OC, such as colorectal and prostate cancers [251] (see **Figure 1-C**). Interestingly, the same report described three carriers of *BRIP1* PVs each having a family history of colorectal cancer or uterine cancer [251]. Carriers of PVs in *BRIP1* have been reported in colorectal cancer cases with a family history of colorectal cancer and other cancer types, or early-onset disease [451,459–464], that were not explained by known colorectal cancer predisposing genes [465]. PVs in *BRIP1* have also been reported in familial and/or early-onset prostate cancer cases not explained by known prostate cancer risk genes [466–470]. These findings suggest that harbouring *BRIP1* PVs increases risk for developing colorectal [471] or prostate cancer [466]. Thus, *BRIP1* PVs may also be involved in conferring risk to a variety of cancers

other than OC or BC, though penetrance has yet to be determined for these other types of cancer.

4.7. Conclusion

In conclusion, we applied a strategy to characterize candidate *BRIP1* variants in BC and OC cases that were initially identified in medical genetics settings, providing evidence for their role in hereditary and sporadic disease in a defined population exhibiting genetic drift and inferred their biological impact applying in cellulo assays. As we have demonstrated in previous studies of other known BC and OC predisposing genes [404], our strategy in investigating the germline of the genetically unique FC population of Quebec has the potential of identifying variants in cancer predisposing genes that may also be relevant to other populations. Our in cellulo assays involving response to cisplatin and PARP inhibitors revealed the potential impact in abrogating protein function for some of the variants, providing insights on their clinical implications that warrant further investigation in patients harbouring *BRIP1* variants. Although penetrance for *BRIP1* variants identified in the FC population has yet to be established, collectively, our findings further support the classification of c.2990_2993del; p.Thr997ArgfsTer6 as LPV, and provides evidence for the reclassification of c.797C>T; p. Thr266Met and c.2087C>T; p.Pro696Leu from missense variants of VUS to LPV.

4.8. Supplementary Materials

See **Appendix VI**.

4.9. Acknowledgment

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4.10. Conflict of interest

The authors declare no competing interests.

4.11. Ethical approval

This project was conducted and approved according to the guidelines of The McGill University Health Centre Research Ethic Board (MP-37-2019-4783) (see **Appendix IX**).

4.12. Funding

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CHAPTER V: Genetic Analyses of DNA Repair Pathway Associated Genes Implicates New Candidate Cancer Predisposing Genes in Ancestrally Defined familial and sporadic Ovarian Cancer Cases

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See **Appendix VII** for the permission from all the co-authors to include this manuscript as Chapter V of my thesis.

5.1. Preface

In the effort to discover new ovarian cancer predisposing genes, few genes have been discovered using a candidate gene approach based on the knowledge of direct interaction of their encoded proteins with the BRCA1 and BRCA2 proteins in the repair of double-stranded DNA breaks via the homologous recombination pathway.

Therefore, in this chapter, I applied strategies to: (1) identify new candidate risk variants in genes involved in various DNA repair pathways using whole exome sequencing and bioinformatics analyses of the germline of ovarian cancer families of French Canadian ancestry of Quebec, who I confirmed were negative for pathogenic variants in *BRCA1*, *BRCA2, BRIP1, RAD51C* and *RAD51D*; and (2) determine the spectrum and prevalence of these candidate risk variants in ovarian cancer cases and controls of French Canadian ancestry.

5.2. Abstract

Purpose: To determine if there are new rare germline, pathogenic variants in DNA repair pathway genes implicated in hereditary ovarian cancer (OC). **Methods:** A candidate gene approach involving various DNA repair pathway genes was applied to identify rare recurring pathogenic variants in familial OC cases not associated with known OC risk genes from a population exhibiting genetic drift. Whole exome sequencing (WES) data of 15 OC cases from 13 families tested negative for pathogenic variants in known OC risk genes were investigated for candidate variants in 468 DNA repair pathway genes. Filtering and prioritization criteria were applied to WES data to select top candidates for further analyses. Candidates were genotyped in ancestry defined study groups of 214 familial and 998 sporadic OC or breast cancer (BC) cases and 1025 population-matched controls and screened for additional carriers in 605 population-matched OC cases. The candidate genes were also analyzed in WES data from 937 familial or sporadic OC cases of diverse ancestries. **Results:** Top candidate variants in *ERCC5*, *EXO1*, *FANCC, NEIL1* and *NTHL1* were identified in 5/13 (39%) OC families. Collectively, candidate variants were identified in 7/435 (1.6%) sporadic OC cases and 1/566 (0.2%) sporadic BC cases versus 1/1025 (0.1%) controls. Additional carriers were identified in 6/605 (0.9%) OC cases. Tumour DNA from *ERCC5, NEIL1* and *NTHL1* variant carriers exhibited loss of the wild-type variants. Carriers of various candidate variants in these genes were identified in 31/937 (3.3%) OC cases of diverse ancestries versus 0-0.004% in cancer-free controls. **Conclusion:** The strategy of applying a candidate gene approach in a population exhibiting genetic drift identified new candidate OC predisposition variants in DNA repair pathway genes.

Key words: Germline variants; Familial ovarian cancer; Cancer predisposing genes; Whole exome sequencing; DNA repair pathways; single-stranded DNA repair genes; double-stranded DNA repair genes.

5.3. Introduction

Since the identification of *BRCA1* [180] and *BRCA2* [181] over twenty years ago as breast cancer (BC) and ovarian cancer (OC) predisposing genes, involved in the homologous recombination (HR) DNA repair pathway [151], no other major high risk

gene has been reported to account for the remaining familial cancer cases found to be negative for pathogenic variants (PVS) in these genes [168]. Nonetheless, carriers of PVs in *MLH1, MSH2*, *MSH6* or *PMS2* genes involved in the mismatch repair (MMR) pathway [151] have also been shown to have a significantly increased lifetime risk of developing OC [224,261]. However, these carriers are rare cases often associated with hereditary non-polyposis colorectal cancer syndrome families [109]. Indeed, carriers of variants in MMR genes account for fewer than 1% of sporadic OC cases, which is significantly lower than the 5-15% carrier frequency of PVs in *BRCA1* and *BRCA2*, depending on the population studied [100]. Carriers of rare PVs in new OC predisposing genes have been reported such as *RAD51C* [231], *RAD51D* [232] and *BRIP1* [234], genes also involved in the HR DNA repair pathway [151]. The carrier frequency of PVs in each of these genes combined is estimated to be less than 2% of sporadic OC cases [33,100,240,241]. PVs in other DNA repair genes such as *PALB2* [68,255,259,260,285], *CHEK2* [260,472] and *ATM* [259,260], all associated with BC risk, were recently associated with OC, though risk has yet to be established. Other genes also playing a role in various DNA repair pathways have been proposed as candidate OC risk genes such as *FANCM* [68,473], *POLE* [474], *MRE11* [259,475], *RAD1* [259] and *FANCI* [384], and collectively, the frequency of carriers of PVs in these genes are also low relative to *BRCA1* and *BRCA2* carriers. Thus, research has consistently shown that a candidate gene approach investigating DNA pathway genes has successfully identified new and candidate OC predisposing genes [179,303], though it is expected that the carrier frequency is significantly lower relative to carriers harbouring such variants in *BRCA1* and *BRCA2*.

Defining the contribution of moderate- to high-risk genes in OC remains a challenge as it is not clear that all monogenic cancer predisposing genes have been identified for this genetically heterogeneous disease [168]. Based on and the family history of cancer and the population investigated, the proportion of OC families known to be negative for *BRCA1* or *BRCA2* PVs has a wide range of approximately 15-65% [100,190]. Indeed, a recent whole exome sequencing (WES) study of familial and sporadic OC cases revealed significant heterogeneity of candidate OC risk genes, representing diverse functional pathways with relatively few involved in the

approximately 200 investigated DNA repair genes [259]. However, this study focused only on investigating rare, protein-coding loss-of-function (LoF) variants [259]. As there are at least 400 known or putative genes that are directly or indirectly involved in repairing DNA [66,151,476–479], it is plausible that PVs (LoF or missense) in genes not previously investigated in OC could be associated with OC risk that have yet to be identified. As carriers of new candidate variants are likely to be rare, identifying them will be challenging.

We have proposed a strategy for identifying candidate variants in new cancer predisposing genes that involves the investigation of cancer families from populations exhibiting genetic drift [182]. Over time, rare PVs in such populations could attain disproportionally high carrier frequencies of rare risk variants relative to the general population [319,320]. For example, *PALB2* [244] and *BRIP1* [234] were discovered as BC and OC predisposing genes by investigating the germline of cancer families and cases from the Finnish and Icelandic populations, respectively. Our research of French Canadians (FC) from the Quebec population of Canada, identified *RECQL* [321] and *FANCI* [384] as new candidate BC or OC predisposing genes, respectively. Genetic drift in the FCs of Quebec has been attributed to common ancestors as a result of the geographic isolation and multiple waves of expansion of European settlers from France since 1608 [182,319,320,322,327]. Investigating these populations also facilitates the characterization of deleterious variants in known or candidate cancer predisposing genes as all types of variants could be investigated and not only LoF variants [182]. A small number of PVs in *BRCA1* and *BRCA2* [344,347] and one in *PALB2* [366], *RAD51C* [404] and *RAD51D* [342,404] have been shown to be frequently occurring in FC OC and/or BC cases versus population-matched controls. Specific PVs in *MLH1* [363]*, MSH2* [363] and *MSH6* [365] have also been reported in FCs in the context of hereditary non-polyposis colorectal cancer.

We recently reported that not all remaining *BRCA1* and *BRCA2* negative families with at least two close relatives with OC from the FC population of Quebec were due to PVs in *RAD51C*, *RAD51D* or *BRIP1* approximating 40% of familial OC cases unaccounted for by known or emerging OC predisposing genes [182,404,417]. Also, we reported that likely pathogenic variants (LPV) in *FANCI*, a proposed new OC

predisposing gene from the Fanconi anemia (FA) pathway was rarely implicated in familial and sporadic OC cases in this population [384]. We posit that DNA repair pathways genes have not been fully explored as candidate OC risk genes. In this study, we report the identification of candidate LPVs in DNA repair pathway genes that were identified by applying a candidate gene approach focusing on an extensive list of 468 DNA repair pathway genes in available WES data derived from the germline of FC familial OC cases. Candidate variants prioritized based on our bioinformatic analyses were selected for targeted genotyping in larger groups of defined FC cases to determine carrier frequencies in 1212 FC and 937 non-FC familial and sporadic OC cases and population-matched controls. Available tumour DNA from our FC carriers also was investigated for loss of the wild-type allele of candidate loci.

5.4. Methods

5.4.1. Study participants

FC cancer cases and controls are described in **Figure 5.1.** and **Table S5.1**. For the discovery of new candidate OC risk variants (study phase I; **Figure 5.1-A**), WES data from peripheral blood lymphocytes (PBL) DNA was available from 15 OC cases from 13 families, each family having at least two first-, second- or third-degree relatives with OC. These cases were confirmed being negative for PVs in the known OC risk genes: *BRCA1*, *BRCA2, BRIP1, RAD51C* or *RAD51D* as previously reported [404,417]. This group includes three index cases harbouring a LPV in *FANCI* c.1813C>T; p.Leu605Phe [384]. As *FANCI* remains a candidate OC predisposing gene requiring further independent studies, we did not exclude *FANCI* variant carriers from any of our study groups for our investigation.

Targeted analyses of the candidate variants was performed to determine their carrier frequencies (study phase II; **Figure 5.1-B**) on the PBL DNA from FC OC cases, regardless of their carrier status for *BRCA1* and *BRCA2* PVs, from 42 hereditary breast and ovarian cancer (HBOC) syndrome families having one OC and at least two with BC cases in the same familial branch and 435 sporadic cases not selected for age at diagnosis with the disease or for family history of any cancers. Genetic data was available from 1025 population-matched controls provided by three independent

biobanks as previously described [404]. As known OC predisposing genes are also involved in BC risk (nccn.org/guidelines/category_2), targeted analyses of the candidate variants was also performed on the PBL DNA from FC BC cases, regardless of their *BRCA1* and *BRCA2* PVs carrier status, from 33 HBOC families, 139 hereditary breast cancer (HBC) syndrome families having at least three close relatives with BC from the same familial branch and 563 sporadic cases not selected for age at diagnosis with the disease or for family history of any cancers.

Targeted analyses of PBL DNA from additional OC cases was performed to identify more OC carriers of our candidate variants (study phase III; **Figure 5.1-C**). These groups were comprised of: 52 sporadic early-onset cases diagnosed with high-grade serous ovarian carcinoma (HGSC) before the age of 50 years who tested negative for PVs in *BRCA1* or *BRCA2*; and 553 OC cases, regardless of their *BRCA1* and *BRCA2* PVs carrier status and not defined by any criteria as previously described in this study [404].

The majority of FC cancer cases self-reported FC ancestry of Quebec as described previously [344,345,347,352,384,404,417,480–484]. FC controls from Université de Sherbrooke-The Genetics of Glucose Regulation in Gestation and Growth (Gen3G) [410] and McGill University-Montreal Neurological Institute (MNI) [411] biobanks self-reported FC ancestry as described previously [404]. FC controls from CARTaGENE biobank (cartagene.qc.ca) were born in Quebec, reported being FC ancestry, having parents and all four grandparents born in Canada and French as first language learned as described previously [404,409].

The cancer cases and controls not selected for being of FC ancestry of Quebec, mainly of European ancestry are referred to as non-FC groups in this study, were available from different resources. Genetic analyses to determine the spectrum and prevalence of candidate variants in genes that were identified in the study phase I were performed (study phase IV; **Figure 5.1-D**) on available genetic data derived from PBL DNA from three independent groups with OC: 9 OC cases from 7 families with at least two close relatives with OC (MIX familial OC cases) [417]; 516 OC familial or sporadic cases from the Australian population (AUS OC cases) [259] and 412 OC cases as part of the Pan-Cancer – The Cancer Genome Atlas (TCGA) project (not selected for

ethnicity) [407] and cancer-free controls as part of the Genome Aggregation Database (gnomAD) v2.1.1. [413].

All biological samples, clinico-pathological, pedigree and relevant medical genetic information from the cancer cases and control groups that were investigated in this study are from biobanks where participants had been recruited in accordance with ethical guidelines of the biobanks respective Institutions Research Ethics Boards as described in **Table S5.1**. Where applicable, samples anonymized at source by the providers, were assigned a unique identifier (PT followed by four digits) to further protect their identity. This project was conducted with approval and in accordance with the guidelines of The McGill University Health Centre Research Ethics Board (MP-37-2019-4783).

5.4.2. *Identifying and selecting for top candidate variants in FC cancer cases*

For phase I of the study (**Figure 5.1.**), WES data was available from PBL DNA from 15 OC index cases from 13 cancer families that had at least one first-, second- or third-degree relative from the same familial branch with OC, and were confirmed being negative for PVs in *BRCA1*, *BRCA2, BRIP1, RAD51C* or *RAD51D* by WES analyses [404,417]. WES had been subjected to a customized bioinformatics pipeline for germline variant calling at the McGill Genome Center as previously reported by our group [384,404]. In brief, NimbleGen SeqCap® EZ Exome v3.0 library kit (Roche, US), followed by paired-end sequencing on different Illumina HiSeq platforms was performed. Reads were aligned to the human reference genome assembly GRCh37/hg19 using Burrows-Wheeler aligner v0.7.17, followed by PCR deduplication using Picard v2.9.0. Realignment around small insertions and deletions was performed, and germline variants were called using HaplotypeCaller using Genome Analysis Toolkit (GATK) v3.5. Variants were then filtered for base sequencing quality score ≥30 and annotated using Ensembl Variant Effect Predictor (VEP) and GEMINI v0.19.1.

Using a candidate gene approach, a curated list of 468 known or putative DNA repair genes [66,476–479] (ebi.ac.uk/QuickGO/term/GO:0006281) (**Table S5.2.**) were investigated for candidate PVs in WES data from selected index OC cases (**Figure 5.1.**). Variants identified in these DNA repair genes were extracted from the annotated

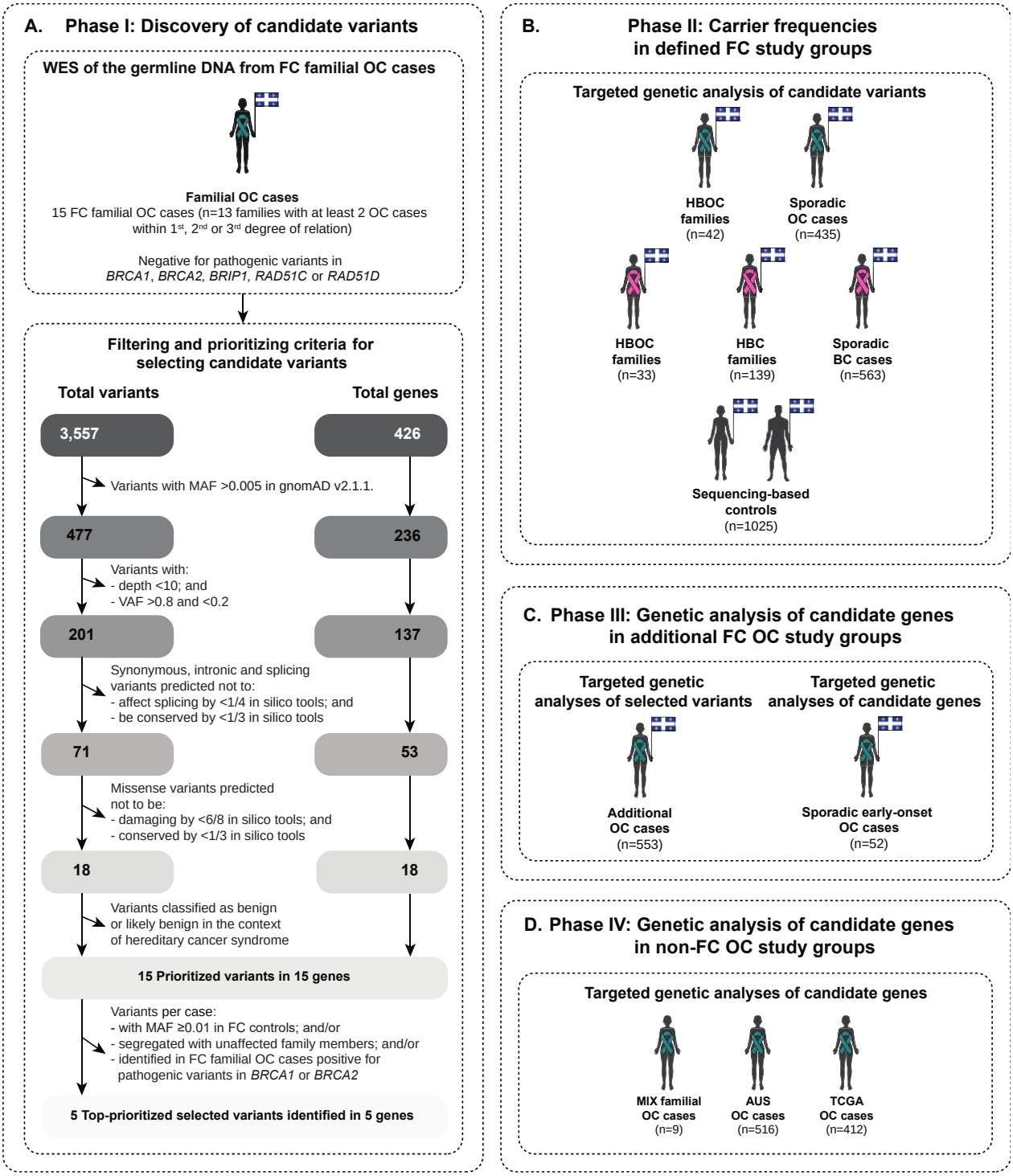

Figure 5.1. Scheme describing different phases of this study in identifying and evaluating candidate variants in genes involved in various DNA repair pathways. The diagram illustrates: (A) study phase I for identifying candidate variants by applying a candidate gene approach of known or putative DNA repair genes (see **Table S5.2.**) on

the germline DNA from familial ovarian cancer (OC) cases of French Canadians (FC) of Quebec by whole exome sequencing (WES) and bioinformatics analyses (see **Table S5.1.**); (B) study phase II for determining the carrier frequencies of the top-prioritized candidate variants in FC familial and sporadic OC and BC cases, including hereditary breast and ovarian cancer (HBOC) syndrome and hereditary breast cancer (HBC) syndrome families, and population-matched controls by targeted genetic analyses (see **Table S5.1.**); (C) study phase III for identifying additional carriers in FC OC cases by targeted genetic analyses (see **Table S5.1.**); and (D) study phase IV for identifying candidate variants in the identified candidate DNA repair genes from phase I in non-FC OC cases, mainly of European origin, by targeted genetic analyses: (MIX: mixed ethnicity; AUS: Australian; and TCGA: The Cancer Genome Atlas) (see **Table S5.1.**). Teal ribbon signifies women with OC and pink ribbon signifies women with BC, and diagrams contain the provincial flag of Quebec, Canada denoting the geographic ascertainment of cases and controls. MAF: Minor allele frequency; and VAF: Variant allele frequency.

variant call files (VCF) from the index OC cases (**Figure 5.1.)**. Variants with minor allele frequency (MAF) >0.005 in gnomAD v2.1.1. [408,413], with total low coverage <10 reads and/or those with variant allele frequency (VAF) <0.2 and >0.8 were filtered out and retained variants were subjected for further prioritization and selection. These thresholds have been tested previously under the assumption that new variants follow an autosomal dominant mode of inheritance [485]. These variants were then verified by manual inspection in the aligned sequences in compressed binary alignment map (BAM) files by Integrative Genomics Viewer (IGV) v2.4.10. [486].

Top candidate variants were selected from this master list of variants for further analyses based on various prioritization criteria as shown in **Figure 5.1-A**. First, we prioritized LoF variants (nonsense, frameshift and alternative splicing variants), inframe, missense and intronic variants, which were predicted to be conserved and damaging at the RNA or protein level by 15 selected in silico tools: (1) by at least one out of three prediction tools for conservation Genomic Evolutionary Rate Profiling v1.0 (GERP++ [score≥2.0]) [422], Phylogenetic P value of 100 vertebrates v4.2 (PhyloP 100 way [score

≥0.2]) [423] and PHAST Conservation of 100 vertebrates v4.2 (PhastCons 100 way [score ≥0.9]) [424]; (2) by at least one out of four prediction tools for splicing Maximum Entropy Estimates of Splice Junction v2.0 (MaxEntScan) [425], two different Database Splicing Consensus Single Nucleotide Variant (dbscSNV) in silico tools: AdaBoost v4.0 (ADA [score ≥0.4]) and Random Forest v4.0 (RF [score≥0.4]) [487] and SpliceAI (score ≥0.4) [488]; and (3) at least six out of eight prediction tools for damaging of protein function based on their best performance [418,420,421,489]: Combined Annotation Dependent Depletion v1.4 (CADD [Phred score ≥20]) [433], Eigen (score ≥0.0) [432], Meta-analytic Logistic Regression v4.2 (MetaLR [score ≥0.5]) [429], Meta-analytic support Vector Machine v4.2 (MetaSVM [score ≥0.0]) [429], MetaRNN 4.2 (score ≥0.5) [430], Rare Exome Variant Ensemble Learner v4.2 (REVEL [score ≥0.5)) [428], Variant Effect Scoring Test v4.2 (VEST [score ≥0.5]) [431] and Protein Variation Effect Analyzer v4.0 (PROVEAN v4.0 [score ≤−2.5]) [434]. Then, the variants having a clinical classification as benign or likely benign in the context of hereditary cancer syndromes in ClinVar [414,415] and/or American College of Medical Genetics and Genomics (ACMG) guidelines [416,449] were given a lower priority for further investigation.

The remaining prioritized variants were then subjected to further prioritization. Variants were surveyed in available genetic data generated from the germline of three FC study groups (**Table S5.1.**): (1) WES data from the germline of 52 sporadic early-onset OC cases negative for PVs in *BRCA1* and *BRCA2* [404,417]; (2) WES data from the germline of 24 index OC cases from HBOC families positive for PVs in *BRCA1* and *BRCA2* [417]; and (3) sequencing-based (WES or whole genome sequencing [WGS]) data and/or genotyping-based data from 1025 FC controls [404]. Then, the variants were subjected for further selection and characterization for genetic analyses.

Selected top candidate variants were verified in the PBL DNA by bidirectional Sanger sequencing using customized primers (available upon request) performed at the McGill Genome Center as described previously [182,289,384,404,417]. Sequencing chromatograms were visually inspected for variant heterozygosity using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer institute, Amsterdam, The Netherlands).

5.4.3. Determining carrier frequencies of selected candidate variants in FC cancer cases and controls

Selected top candidate variants were investigated for carrier frequencies in defined FC study groups (study phase II) comprised of 42 index OC and 33 index BC cases from 75 HBOC families, 139 index BC cases from 139 HBC families, 435 sporadic OC cases and 563 sporadic BC cases (**Figure 5.1-B** and **Table S5.1.**). PBL DNA from index cases were genotyped using customized TaqMan® [436], Sequenom iPLEX® Gold [437] or Fluidigm® SNP Type™[438] genotyping assays (primers available upon request) as described previously [381,384,404]. Tumour DNA samples from the index case were genotyped where PBL DNA was no longer available from the biobank. Carriers of candidate variants were verified by bidirectional Sanger sequencing of PBL DNA as described above. Selected candidate variants were also investigated for carrier frequency in population-matched controls by surveying 1025 available sequencing-based data sets (**Table S5.1.**) and/or 8493 single nucleotide polymorphism (SNP) genotyping-based as previously described [404]. For probes of variants not presented on the SNP array, pre-phasing and imputation were performed as described previously [384,404].

Pair-wise comparisons were performed of carrier frequencies of candidate variants in different FC cancer groups versus sequencing-based controls. Two-tailed Fisher's exact test was used to compare carrier frequencies in the cancer versus control groups where un-adjusted *P* values <0.05 for multiple testing was considered significant.

5.4.4. Targeted genetic analyses of selected candidate variants or genes in FC cancer cases

To further characterize our candidate variants and genes in a population exhibiting genetic drift, we investigated carrier status in additional OCs from the FC population. Selected top candidate variants were investigated (study phase III) in 52 sporadic early-onset FC HGSC cases and in an additional 553 FC OC cases by surveying available genetic data or targeted genotyping of PBL DNA (**Figure 5.1-C** and **Table S5.1.**). We also investigated for other variants in our gene candidates that met our filtering and

prioritizing criteria in the available WES data from the sporadic early-onset OC cases (**Figure 5.1-C**).

5.4.5. Loss of heterozygosity analyses of candidate genes loci in OC tumour DNA from FC candidate variant carriers

To investigate evidence for inactivation of candidate genes in cancer cells, we performed loss of heterozygosity (LOH) analysis of tumour DNA from variant carriers. Bi-directional Sanger sequencing of available tumour DNA was performed using customized primers (available upon request) as described above. Extracted DNA from fresh-frozen (FF) or histopathological sections from formalin-fixed paraffin-embedded (FFPE) tumour tissues were provided by the RRCancer biobank for DNA extraction and LOH analysis (Promega, Canada). Sequencing chromatograms were inspected for loss of the wild-type alleles using 4Peaks v1.8. (nucleobytes.com/4peaks/) (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

5.4.6. Genetic analyses of candidate genes in non-FC cancer cases and controls

To further characterize the candidate variants and genes identified in our FC cancer cases, we investigated available genetic data from other populations that were not specifically selected for FC ancestry. The spectrum and prevalence of our candidate variants were investigated in genetic data from non-FC OC cases of being predominantly of European ancestry and cancer-free controls were investigated for new variants in our candidate genes that met our filtering and prioritizing criteria (**Figure 5.1-D** and **Table S5.1.**). Variants were extracted from the annotated VCF files generated by WES data from the germline of: (1) 9 MIX familial OC cases; (2) 516 AUS OC cases; and (3) 412 OC cases from the Pan-Cancer TCGA project. Variants were extracted from the comma separated value (CSV) files downloaded directly from (https://gnomad.broadinstitute.org). All variants were annotated and subjected to our filtering and prioritizing criteria to identify candidate variants as described above (see **Figure 5.1-A**).

5.4.7. Genetic analyses for co-occurring rare pathogenic variants in known OC risk genes in FC and non-FC candidate variant carriers

We investigated available WES data from OC cases, to determine whether the identified carriers of candidate variants (regardless of the ethnicity and study phase in which they were identified) also harbour rare PVs or LPVs in known OC risk genes (n=11): *BRCA1* (NM_007294.4) and *BRCA2* (NM_000059.4), *MLH1* (NM_000249.4), *MSH2* (NM_000251.3), *MSH6* (NM_000179.3), *PMS2* (NM_000535.7), *BRIP1* (NM_032043.3), *RAD51C* (NM_058216.3), *RAD51D* (NM_001142571.2), *PALB2* (NM_024675.4) and *ATM* (NM_000051.4) based on the National Comprehensive Cancer Network (NCCN) Clinical Practice in Oncology Guidelines 2022 (Version 2.2022) —Genetic/Familial High-Risk Assessment: Breast, Ovarian and Pancreatic (nccn.org/guidelines/category_2). Variants were extracted from the annotated VCF files from carriers and subjected to our filtering and prioritizing criteria as previously described [417].

5.5. Results

5.5.1. Prioritization and selection of candidate variants: Phase I

We first extracted variants identified in a curated list of 468 DNA repair genes (**Table S5.2.**) from VCF files generated from WES data from the 15 index FC OC cases from 13 families. In these index cases, we identified a total of 3,557 variants in 426 of 468 DNA repair genes (**Figure 5.1.**). Based on their rarity and quality, we retained a total of 201 variants in 137 of 426 DNA repair genes where each index case harboured 3 to 25 (median=15) such variants. From this list of 201 variants, we prioritized candidates that were predicted to be conserved or damaging at the level of RNA or protein using our selected in silico tools; and those classified benign or likely benign in the context of hereditary cancers using ClinVar and/or ACMG guidelines were not pursued further. Using these criteria, we retained a total of 15 variants of the 201 variants, each of which was found in a different gene: 3 nonsense variants, 1 canonical alternative splicing variant, 1 inframe and 10 missense variants (**Table S5.3.**). These variants were identified in 10 of the 15 index cases from 8 out of the 13 OC families (**Figures 5.2.** and **S5.1.**). One of these variants was identified in two OC cases from the same family, two variants were identified in two OC cases from the same family and the remaining 12

variants were identified in one index case from independent OC families (**Table S5.3.**). Two cases harboured either three or four variants, while the remaining nine cases harboured one to two variants.

To select our top candidates for further analyses, we reviewed the individual context wherein the 15 variants were identified as shown in **Table S5.3.** and **Figures 5.2.** and **S5.1**. We estimated the allele frequencies of the 15 candidate variants in population-matched FC controls. Thereby, we did not pursue *ALKBH3* c.677A>G; p.Asn226Ser, which was identified in an index case (PT0136) from family F1506, as it has a MAF ≥0.01 in the FC controls. We also excluded the missense variant in *DNA2* c.836C>T; p.Thr279Ile for further analyses as it was identified in the index case (PT0128) and four unaffected members of the family F694, and was not inherited from the affected mother with the family history of OC and other cancers (**Figure S5.1.**). We did not pursue *RBBP8* c.1941T>G; p.Asp647Glu as it was not harboured by the other index OC case (PT0056) from the same family F1528 (**Figure S5.1.**). Additional variants that were excluded for further analyses included: (1) *RHNO1* c.250C>T; p.Arg84Ter in the index OC case (PT0158) from family F1288; (2) *ATRX* c.4377_4379del; p.Glu1464del in one of the index OC case (PT0057) from family F1528 as they were classified as benign in ClinVar and by ACMG guidelines, and the latter as not being harboured by the other index OC case of the same family F1528 (**Figure S5.1.**); (3) *SMARCA2* c.3265C>T in the index OC case (PT0128) from family F694; p.Arg1089Trp; and (4) *KMT2C* c.6916C>T; p.Pro2306Ser in the index OC case (PT0047) from family F1490 as variants in these genes are associated with non-cancer related syndromes (**Figure S5.1.**). Heterozygous germline variants in *SMARCA2* are linked with Nicolaides-Baraitser syndrome (MIM: 601358), which is characterized by mental retardation, seizures, limited to absence of speech ability, short stature, dysmorphic facial features and sparse hair [490–496]; and heterozygous germline variants in *KMT2C* are linked with Kleefstra syndrome, type 2 (MIM: 617768), which is characterized by delayed psychomotor development, variable intellectual disability and mild dysmorphic features [497–500]. A genotype-phenotype of heterozygous variants located within exon 15-25 of *SMARCA2*, which encodes the ATPase domain, have been recently reported; this report also revealed that over 80% of these variants were de novo based on WES analyses of

80 cases in trios with Nicolaides-Baraitser syndrome that have been documented worldwide so far [493,495,496]. Our *SMARCA2* c.3265C>T; p.Arg1089Trp have never been reported in the literature, but it is located in exon 23 that encodes the ATPase domain [493]. Whereas, our *KMT2C* c.6916C>T; p.Pro2306Ser was reported in the context of Kleefstra syndrome [497]. Finally, we did not pursue: *RECQL5* c.918G>A; p.Met306Ile, *ASCC3* c.3808C>T; p.Arg1270Ter and *UBB* c.569C>A; p.Pro190His as all were harboured by the same index case (PT0139) form family F1606 that also harbouring *FANCC* c.897G>T; p.Arg299Ser as this variant is a plausible and intriguing candidate where *FANCC* has been reported as a candidate BC predisposing gene [501]. Thus, *FANCC* c.897G>T; p.Arg299Ser and the remaining variants identified in *ERCC5* c.2556A>G; p.Ile852Met*, EXO1* c.1268-1G>T*, NEIL1* c.248G>T; p.Gly83Asp and *NTHL1* c.244C>T; p.Gln82Ter from our list of most promising variants selected for further analyses as candidates.

5.5.2. Characterization of the selected top candidate variants: Phase I

We selected five variants each identified in an OC family for further characterization and analyses (**Table 5.1.**, **Figures 5.2** and **5.3.**): a nonsense variant *NTHL1* c.244C>T; p.Gln82Ter*,* a canonical splicing variant *EXO1* c.1268-1G>T, an exonic splicing variant *FANCC* c.897G>T; p.Arg299Ser and two missense variants *ERCC5* c.2556A>G; p.Ile852Met and *NEIL1* c.248G>T; p.Gly83Asp. Both missense variants were predicted to affect amino acid residues that are located in catalytic domains of their respective proteins that are critical to the biological function of ERCC5 [502] and NEIL1 [503] in the HR, nucleotide excision repair (NER) and base excision repair (BER) pathways.

Except for *EXO1* c.1268-1G>T, which was not found in the gnomAD v2.1.1. database, all other candidate variants were found to have MAFs between 0.002 and 0.00001 in the non-cancer non-Finnish European populations with variation in these frequencies across populations of different ancestry groups (**Table S5.4.**). The loci of all five candidate variants were predicted to be conserved by at least one of the selected in silico tools. The variants in *EXO1* and *FANCC* were predicted to affect splicing by the four selected in silico tools. The missense variants in *ERCC5* and *NEIL1* were predicted to be damaging by at least six selected in silico tools, including REVEL and VEST,

which are two of the recently validated as top performing prediction in silico tools [421] (**Table S5.3.**). Only *NTHL1* c.244C>T; p.Gln82Ter is classified as PV in ClinVar and by ACMG guidelines in the context of hereditary multi-cancer syndrome in an autosomal recessive mode of inheritance, and has recently been associated with BC risk in an autosomal dominant mode of inheritance [504,505]. Whereas, *FANCC* c.897G>T; p.Arg299Ser was classified as being of uncertain significance (VUS) in ClinVar in the context of FA, an autosomal recessive disorder (MIM: 227645) and as LPV by ACMG guidelines. As noted above, *FANCC* has been associated with BC predisposition in an autosomal dominant mode of inheritance [501]. The remaining candidate variants have not been reported in ClinVar, but classified by ACMG guidelines as LPV for *EXO1* c.1268-1G>T, VUS for *ERCC5* c.2556A>G; p.Ile852Met and likely benign for *NEIL1* c.248G>T; p.Gly83Asp.

We genotyped PBL DNA samples from family members of the index carriers where possible to determine if the candidate allele segregated with disease (**Figure 2**). For family F1085, both unaffected mother (PT0178) and sister (PT0177) of the index carrier cases did not carry *NTHL1* c.244C>T; p.Gln82Ter, suggesting that the variant allele may have been transmitted paternally. This observation is interesting as the paternal side of the family had numerous cancer cases including BC, OC, colorectal and pancreatic cancers. In family F1288, though the mother of the index *EXO1* c.1268-1G>T carrier case (PT0158) with BC and melanoma also carried the *EXO1* variant, her sibling (PT0180) and maternal female cousin (PT0181) both with BC were not carriers of the *EXO1* variant. These observations are interesting given the number of different types of cancer cases on the maternal side of the family. In family F1506, the index carrier harbouring *ERCC5* c.2556 A>G also had a remarkable family history of diverse cancer types, whereas the index carrier of *NEIL1* c.248G>T; c.1268-1G>T from family F1601 had a cancer family history consistent with HBOC syndrome. The index carrier of *FANCC* c.897G>T; p.Arg299Ser from family F1606 reported a mother with OC and a father with lung cancer.

Table 5.1. Characteristics of top-prioritized candidate variants identified in familial French Canadian cases with ovarian cancer.

Genomic features (hg19/GRCh37)[1]					
Gene	*NTHL1*	*EXO1*	*FANCC*	*ERCC5*	*NEIL1*
Transcript	NM_002528.7	NM_130398.4	NM_000136.3	NM_000123.4	NM_024608.4
Cytoband	16p13.3	1q43	9q22.32	13q33.1	15q24.2
Genome change	g.2096239G>A	g.242035333G>T	g.97887467C>G	g.103520485A>G	g.75641494G>A
Coding change	c.244C>T	c.1268-1G>T	c.897G>T	c.2556A>G	c.248G>T
Protein change	p.Gln82Ter	-	p.Arg299Ser	p.Ile852Met	p.Gly83Asp
Allele frequencies in gnomAD[2]					
Non-cancer non-Finnish European	0.002 (235/118138)	-	8.8e-06 (1/113756)	9.74e-06 (1/102714)	0.001 (140/117290)
Clinical classification[3]					
ClinVar (number of submissions)	PV (6); VUS (1)	-	VUS (2)	-	-
ACMG guidelines (implemented rule)	PV (PVS1-PP5-PM2-PP3)	PV (PVS1-PM2-PP3)	LPV (PVS1-PM2-PP3)	VUS (PM2-PP3)	LB (BS1-BP1-BP6-PP3)
Predictions by in silico tools[4]					
GERP++ v1.0	Conserved	Conserved	Conserved	Conserved	Conserved
PhyloP 100 way v4.2	Not conserved	Conserved	Not conserved	Not conserved	Conserved
PhastCons 100 way v4.2	Not conserved	Conserved	Not conserved	Conserved	Conserved
REVEL v4.2	-	-	Benign	Pathogenic	Pathogenic
MetaLR v4.2	-	-	Tolerated	Tolerated	Damaging
MetaSVM v4.2	-	-	Tolerated	Tolerated	Damaging
MetaRNN v4.2	-	-	Damaging	Damaging	Tolerated
CADD v1.6	Damaging	Damaging	Damaging	Damaging	Damaging
VEST v4.2	-	-	Damaging	Damaging	Damaging

EIGEN PC v4.2	-	-	Pathogenic	Pathogenic	Pathogenic
PROVEAN v4.2	-	-	Damaging	Damaging	Damaging
ADA v1.1	-	Affecting splicing	Affecting splicing	-	-
RF v1.1	-	Affecting splicing	Affecting splicing	-	-
MaxEntScan v2.0	-	Affecting splicing	Affecting splicing	-	-
SpliceAI	-	Affecting splicing	Affecting splicing	-	-

[1] Annotation of candidate variants based on the National Center for Biotechnology Information (NCBI) - Reference Sequence (RefSeq) database (tark.ensembl.org/web/manelist/) [412]; [2] Allele frequencies in the non-cancer, non-Finnish European controls from the Genome Aggregation Database (gnomAD) v2.1.1 database (gnomad.broadinstitute.org) [413]; [3] Clinical classifications from ClinVar (ncbi.nlm.nih.gov/clinvar/) [414,415] based on last revision in March 2022, and American College of Medical Genetics and Genomics (ACMG) guidelines [416,449]; [4] Applied in silico tools for conservation, damaging or affecting splicing selected based on their best performance [418–420,506]. Classification of variants by ACMG guidelines as: BP1: Benign Supporting Level 1; BP6: Benign Supporting Level 6; BS1: Benign Strong Level 1; LB: Likely Benign; LPV: Likely Pathogenic Variant; PM2: Pathogenic Moderate Level 2; PP3: Pathogenic Supporting Level 3; PP5: Pathogenic Supporting Level 5; PV: Pathogenic Variant; PVS1: Pathogenic Very Strong Level 1; VUS: Variant of Uncertain Significance; and (-): Not applicable/reported.

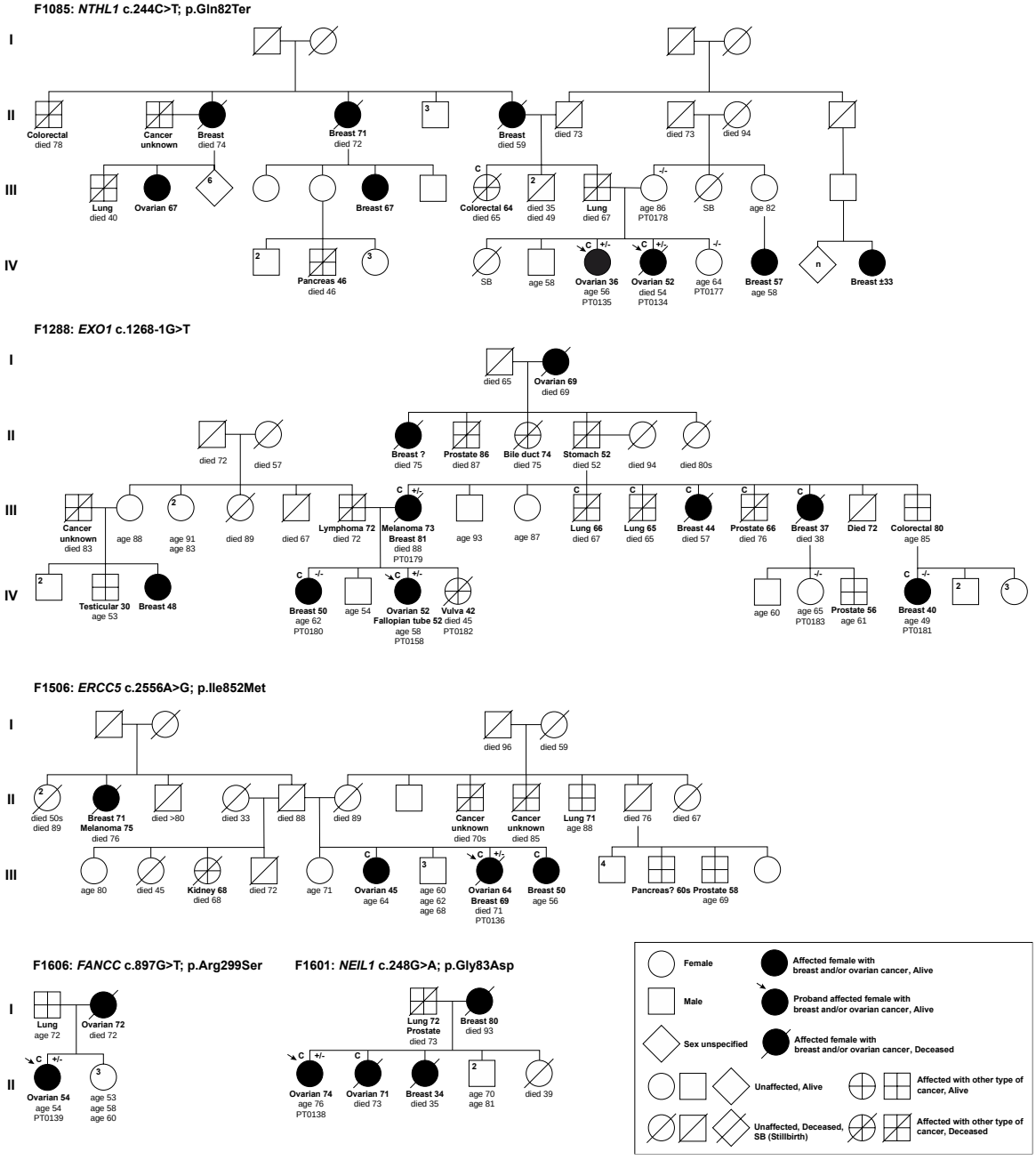

Figure 5.2. Pedigrees of index ovarian cancer cases harbouring candidate variants in DNA repair genes identified in phase I of the study.

Selected top candidate variants were identified in 5 of 13 families having at least two or more OC cases. Anonymized pedigrees indicate carrier status of tested index case (arrow) and available family members denoted by plus (carrier) or minus (not a carrier) signs. All carriers were found in a heterozygous state. Age in years is shown at cancer

diagnosis and death where applicable. Superscript C denotes histological subtypes that were confirmed by pathology reports or death certificates.

5.5.3. Identification of carriers of selected candidate variants in defined FC cancer study groups: Phase II

We genotyped or surveyed available genetic data of our candidate variants: *NTHL1* c.244C>T; p.Gln82Ter, *EXO1* c.1268-1G>T*, FANCC* c.897G>T; p.Arg299Ser, *ERCC5* c.2556A>G; p.Ile852Met and *NEIL1* c.248G>T; p.Gly83Asp in different FC OC and BC study groups and population-matched controls, regardless of their carrier status for *BRCA1* and *BRCA2* PVs (**Figure 5.1-B** and **Table S5.1.**). Carriers were identified in the sporadic OC study group with frequencies of 0.2% (1/435) for *EXO1* variant carriers and 0.5% (2/435) for carriers of each *NTHL1*, *ERCC5* or *NEIL1* variants, and one *NTHL1* carrier among sporadic BC cases (0.2%, 1/563). Carriers were not identified among index cases from HBOC and HBC families. *FANCC* variant carriers were not identified in any of these FC cancer study groups.

Table 5.2. Carrier frequency of candidate variants in French Canadian cancer cases and controls.

Gene	Variant	Study groups	Cancer cases tested	Number of tested participants (or families) per study group	Number of variant carriers (%)	*p*-value
NTHL1	c.244C>T; p.Gln82Ter	HBOC families	OC	42 (42)	0	-
		Sporadic OC cases	OC	435	**2/435 (0.5)**	0.213
		HBOC families	BC	33 (33)	0	-
		HBC families	BC	139 (139)	0	-
		Sporadic BC cases	BC	563	**1/563 (0.2)**	1.000
		FC sequencing-based controls	-	1025	**1/1025 (0.1)**	-
EXO1	c.1268-1G>T	HBOC families	OC	42 (42)	0	-
		Sporadic OC cases	OC	435	**1/435 (0.2)**[2]	0.298
		HBOC families	BC	33 (33)	0	-

		HBC families	BC	139 (139)	0	-
		Sporadic BC cases	BC	563	0	-
		FC sequencing-based controls	-	1025	0	-
FANCC	c.897G>T; p.Arg299Ser	HBOC families	OC	42 (42)	0	-
		Sporadic OC cases	OC	435	0	-
		HBOC families	BC	33 (33)	0	-
		HBC families	BC	139 (139)	0	-
		Sporadic BC cases	BC	563	0	-
		FC sequencing-based controls	-	1025	0	-
ERCC5	c.2556A>G; p.Ile852Met	HBOC families	OC	42 (42)	0	-
		Sporadic OC cases	OC	435	**2/435 (0.5)[2]**	0.213
		HBOC families	BC	33 (33)	0	-
		HBC families	BC	139 (139)	0	-
		Sporadic BC cases	BC	563	0	-
		FC sequencing-based controls	-	1025	0	-
NEIL1	c.248G>T; p.Gly83Asp	HBOC families	OC	42 (42)	0	-
		Sporadic OC cases	OC	435	**2/435 (0.5)[2]**	0.213
		HBOC families	BC	33 (33)	0	-
		HBC families	BC	139 (139)	0	-
		Sporadic BC cases	BC	563	0	-
		FC sequencing-based controls	-	1025	0	-

[1] Two-tailed *p*-values (not adjusted for multiple testing) calculated using Fisher's exact test in pair-wise comparisons between variant carriers in cancer study groups and population-matched controls. BC: Breast cancer; HBC: Hereditary breast cancer syndrome; HBOC: Hereditary breast and ovarian cancer syndrome; OC: Ovarian cancer; and (-): Not applicable.

Carriers of all candidate variants in the FC controls are likely very rare as indicated by the observation that only one carrier was identified among 1025 FC sequencing-based controls (**Table 5.2.**). Identifying a carrier of *NTHL1* c.224C>T; p.Gln82Ter was not surprising given the frequency of carriers of this variant = 0.002 in the non-cancer

non-Finnish European population in gnomAD v2.1.1 (**Table S5.4.**). Overall, the carrier frequencies of our candidates are higher in cancer groups relative to our population-matched controls though the results were not significant (**Table 5.2.**).

We investigated our variants in 8493 non-cancer SNP array genotyping-based controls from cancer-free FC population (see **Table S5.1.**). None of the probes for variants in *EXO1, FANCC* and *ERCC5* were represented on any of the SNP arrays, nor was imputation possible as they were not represented in the Haplotype Reference Consortium (HRC.r1) release panel [404]. However, we were able to determine the carrier frequency of the *NTHL1* (0.2%, 19/8493) and *NEIL1* (0.3%, 24/8493) variants (**Table S5.5.**). The frequencies of these variants are consistent with those in the non-cancer non-Finnish European population in gnomAD v2.1.1. (**Table S5.4.**), though we did not identify any carriers among the FC controls harbouring both *NTHL1* and *NEIL1* candidate variants.

5.5.4. Genetic analyses of other FC OC cases identified additional carriers of candidate variants: Phase III

Given genetic drift exhibited by the FC population that may result in higher frequency of candidate variant carriers with OC [182,319,320,404], we genotyped the germline of PBL DNA from additional 553 FC OC cases, which were recruited to the biobank but did not meet our criteria for the abovementioned defined OC study groups, and surveyed WES data available from 52 early-onset OC cases (**Figure 5.1-C** and **Table S5.1.**). We identified a total of six OC cases harbouring *NTHL1* c.244C>T (n=1), *EXO1* c.1268-1G>T (n=1), *FANCC* c.897G>T (n=1) and *NEIL1* c.248G>A (n=3) (**Table S5.6.**).

5.5.5. Genetic analyses of sporadic early-onset FC OC cases identified other variants in our candidate genes: Phase III

Given the genetic heterogeneity observed in the FC population for rare PVs identified in *BRCA1, BRCA2*, *RAD51C* and *RAD51D* [182,342,404], we surveyed WES data available from 52 early-onset FC OC cases diagnosed at less than 50 years of age (**Figure 5.1-C** and **Table S5.1.**). The rationale for investigating this group is based on the plausibility that carriers of some of the known OC predisposing genes are more likely

to develop OC before age of 60 as it is the median age of diagnosis of this disease in the general population with OC [1,3]. We identified a carrier of a rare variant in *NEIL1* c.569C>A; p.Pro276His that met our filtering and prioritizing criteria (**Table S5.6.**). We genotyped this variant in our defined FC cancer study groups and controls, and we did not identify any other carriers of this *NEIL1* variant or in any of the additional 553 FC OC cases. We could not determine the variant carrier frequency in the 8493 genotyping-based FC cancer-free controls as it was not represented on the SNP array, and we could not impute this variant as it was not available in the HRC.r1 haplotype reference panel.

5.5.6. Evidence of loss of the wild-type alleles in tumour DNA from carriers of candidate variants

As known OC risk genes behave as tumour suppressors where there is loss of the gene function in tumours is be expected [465], we performed LOH analyses to investigate one of the classical mechanisms of inactivation of the loci of our candidate genes: *ERCC5*, *EXO1*, *FANCC*, *NEIL1* or *NTHL1*. We were able to perform LOH analyses on OC tumour DNA from some of our carriers of the candidate variants, which were available from the RRCancer biobank: *NTHL1* c.244C>T; p.Gln82Ter (n=4), *FANCC* c.897G>T; p.Arg299Ser (n=1), *ERCC5* c.2556A>G; p.Ile852Met (n=2) and *NEIL1* c.248G>T; p.Gly83Asp (n=3). Chromatograms of bidirectional Sanger sequencing of OC tumour DNA and case matched normal were inspected for allelic content. We observed partial or complete loss of the wild-type alleles in the tumour DNA from two *NEIL1* variant carriers, two *NTHL1* variant carriers and one *ERCC5* variant carrier (**Table S5.7.**); chromatogram of one example is shown in **Figure 5.3**. Moreover, we observed partial or complete loss of the wild-type alleles in the tumour DNA from the left and the right ovaries from both *NTHL1* variant carriers having bilateral OC. There was no clear evidence for loss of the wild-type allele in the remaining samples from tumour DNA from carriers of *FANCC* or *EXO1*. However, loss of the variant allele was observed in the tumour DNA from *FANCC* and *EXO1* variant carriers (**Table S5.7.**).

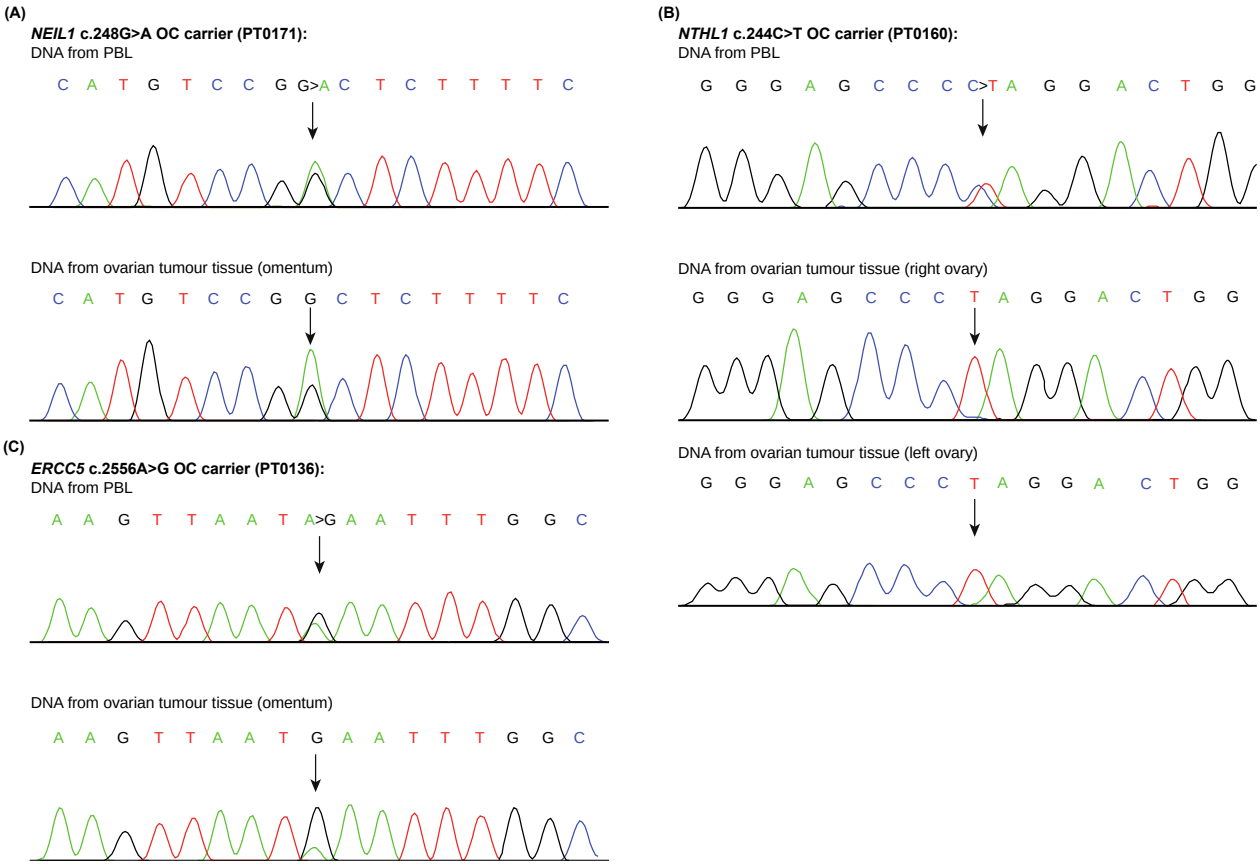

Figure 5.3. Loss of heterozygosity analyses of candidate genes loci.

Sanger sequencing chromatograms showing loss of heterozygosity (LOH) analyses of the candidate variants (see **Table S7**), in genomic peripheral blood lymphocyte (PBL) DNA, ovarian tumour tissue DNA from carriers of (A) *NEIL1* c.248G>T; p.Gly83Asp (PT0171); (B) *NTHL1* c.244C>T; p.Gln82Ter (PT0160); and (C) *ERCC5* c.2556A>G; p.Ile852Met (PT0136). Each variant is indicated by an arrow. One example of such genetic event per candidate variant carrier is shown.

5.5.7. Genetic analyses of non-FC cases identified other candidate variants in our gene candidates: Phase IV

To determine the relevance of our candidate genes to OC in non-FC populations, we investigated the spectrum and prevalence of rare variants in our candidate genes in genetic data from three defined non-FC study groups (**Figure 5.1-D** and **Table S5.1.**). We applied our filtering and prioritizing criteria to WES data that was available from the germline of PBL DNA from: (1) 9 index OC cases from MIX familial OC cases; (2) 516

index AUS OC cases from HBOC and sporadic disease; and (3) 412 OC cases from Pan-Cancer – TCGA. In these study groups, we identified: one of the 9 MIX familial OC cases (11.1%) harbouring a *NTHL1* variant; 17 of 516 AUS OC cases (3.3%) harbouring 11 variants in *NTHL1* (n=6), *NEIL1* (n=3), *ERCC5* (n=1) and *EXO1* (n=1); 12 of 412 Pan-Cancer – TCGA OC (2.9%) harbouring 10 variants in *NEIL1* (n=4), *EXO1* (n=2), *FANCC* (n=2), *NTHL1* (n=1) and *ERCC5* (n=1) (**Table S5.6.** and **Figure 5.4.**). Collectively, these variants are comprised of three nonsense, four frameshift, three alternative splicing and nine missense variants. Variants in and *NEIL1* (n=3), *EXO1* (n=2)*, NTHL1* (n=2), *ERCC5* (n=1) and *FANCC* (n=1) were LoF variants and classified as PVs or LPVs in ClinVar and/or by ACMG guidelines. The remaining variants were missense predicted to be PV or LPVs by our set of in silico tools. Some of these variants were those already identified in our FC study groups: three of 516 (0.6%) AUS OC cases and two of 412 (0.5%) Pan-Cancer – TCGA OC cases carried *NTHL1* c.244C>T; p.Gln82Ter, while one each of 516 (0.2%) AUS OC cases and 412 (0.2%) Pan-Cancer – TCGA OC cases carried *NEIL1* c.248G>A; p.Gly83Asp. We identified one Pan-Cancer – TCGA OC carrier of a synonymous variant in *NEIL1* c.159C>T; p.Gly53Gly that was predicted to affect splicing using SpliceAI [427] that may result in donor gain.

5.5.8. Most candidate variant carriers do not harbour co-occurring pathogenic variants in known OC predisposing genes

We investigated whether OC carriers harbouring any of our candidate variants may also harbour PVs in known OC predisposing genes (n=11) (nccn.org/guidelines/category_2) (**Table S5.8.** and **Table S5.9.**). Only one of the 15 FC OC carriers investigated, carried both *NEIL1* c.248G>A; p.Gly83Asp (PT0175) and *BRCA1* c.5102_5103del; p.Leu1701GlnfsTer14. This *BRCA1* variant is one of the most frequently occurring PVs in OC and BC cases from the FC population [182,344,347,507].

Regarding non-FC OC carriers, one of the 17 OC AUS carriers of *NEIL1* c.248G>A; p.Gly83Asp (PT0314) also harboured a variant in a known OC risk gene *RAD51C* c.145+1_145+2insC which was classified as PV by ACMG guidelines (**Table S5.9.**), this *RAD51C* was reported previously [259]. Two of the 13 carriers of our candidate variants from OC Pan-Cancer – TCGA project cases, harboured variants in

known OC risk genes: a carrier of *NTHL1* c.244C>T; p.Gln82Ter (PT0261) also harboured *BRCA2* c.5065_5066insA; p.Ala1689AspfsTer6, and a carrier of *EXO1* c.2152C>T; p.Gln718Ter (PT0263) also harboured *BRCA2* c.1029del; p.Lys343AsnfsTer6. Both *BRCA2* variants have been classified as PV in the ClinVar database and by ACMG guidelines.

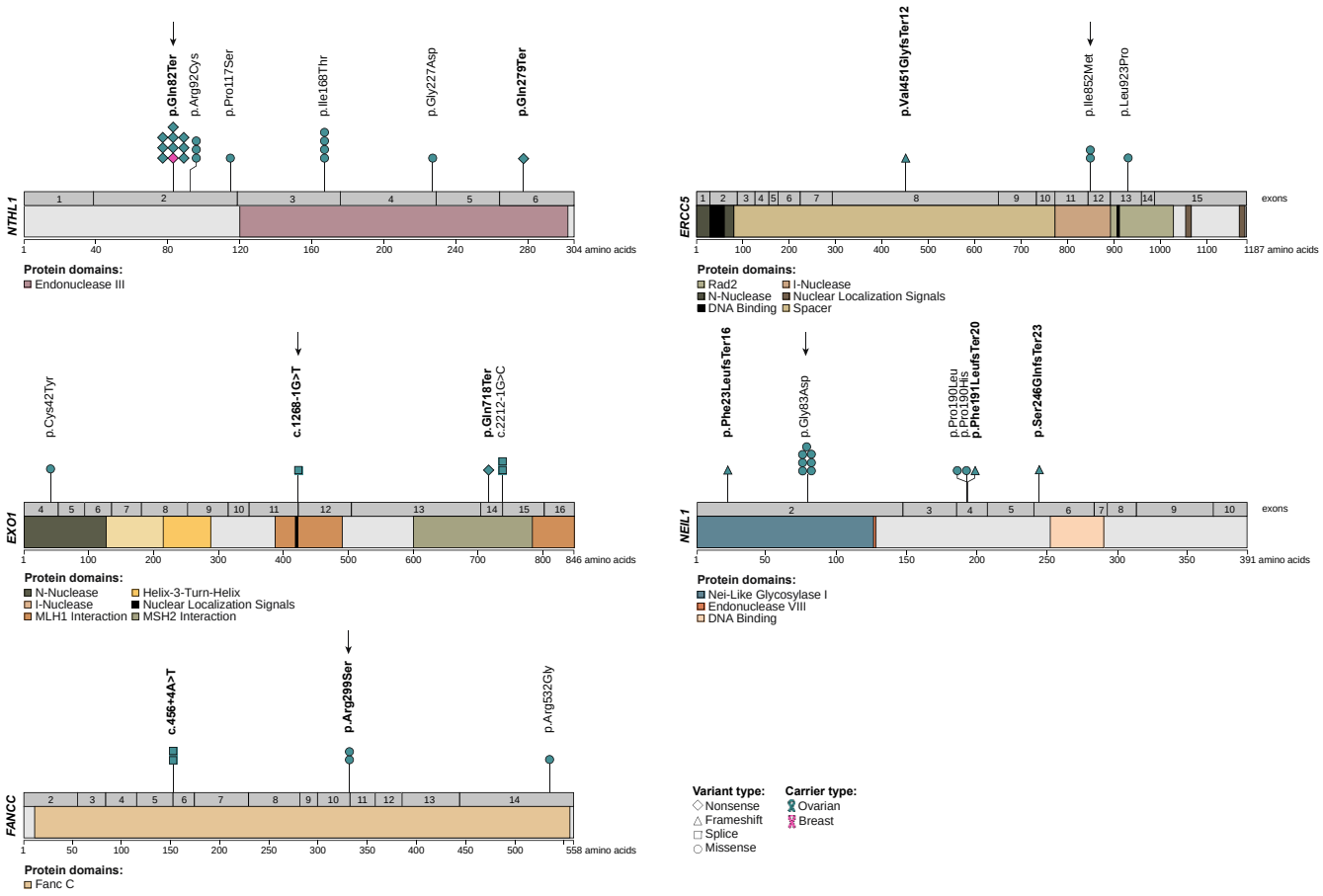

Figure 5.4. Location of candidate variants in *NTHL1*, *EXO1*, *FANCC*, *ERCC5* and *NEIL1* identified in all study groups.

The coding regions and protein domains of candidate genes *NTHL1* (NM_002528.7), *EXO1* (NM_130398.4), *FANCC* (NM_000136.3), *ERCC5* (NM_000123.4) and *NEIL1* (NM_024608.4), based on NCBI RefSeq transcripts (tark.ensembl.org/web/manelist/) [412], were annotated for the location of candidate variants. Variants classified as PV or LPV are bolded and those identified in French Canadians ovarian cancer cases each is indicated with an arrow.

5.6. Discussion

Our investigation of potentially deleterious variants in 468 genes that play a direct or associated role in various DNA repair pathways in the FC population exhibiting genetic drift identified LoF and potentially deleterious missense variants as candidates for OC predisposition in *ERCC5*, *EXO1*, *FANCC*, *NEIL1* or *NTHL1*. Genotyping analyses of independently ascertained FC cancer study groups identified multiple carriers with OC harbouring the same variant, which is likely due to common ancestors within the FC population of Quebec [182,319,320]. Overall, carriers of each variant are rare, each accounting for <1%, but collectively 9.6% of 52 familial OC cases with at least two or more OC cases and 1.6% of all 435 sporadic OC cases versus 0.1% of the population-matched controls. It is notable is that none of the variants were found in known OC predisposing genes (nccn.org/guidelines/category_2), confirming prior findings from either clinical testing or our WES analyses of this group of cases [384,404,417].

Our candidate variants were identified in genes involved in different pathways involved in the repair of single- and/or double-stranded DNA breaks. *ERCC5* is known to be involved in the NER pathway an endonuclease, but it has been shown that this gene is also involved in the BER [508] and HR [502] pathways. *EXO1* is involved in the HR and MMR pathways as an exonuclease [509,510]. *FANCC* plays a role in the FA pathway as a member of the core complex [393]. *NTHL1* and *NEIL1* are DNA glycosylases in the BER pathway [511]. Although the role of these genes in conferring risk to hereditary OC requires further investigation with larger cohorts, a recent Australian study of familial and sporadic OC cases reported that there was a statistically significant difference in the frequency of germline LoF variants in the single-stranded DNA repair pathway genes involved in BER, NER and MMR in OC cases versus non-cancer controls [259]. On the other hand, homozygous or compound heterozygous PVs in *NTHL1* have been linked to Familial adenomatous polyposis - 3 (MIM: 616415) [512] and most recently a multi-tumour phenotype [513]. Homozygous or compound heterozygous PVs in *FANCC* and *ERCC5* are known to be linked to autosomal recessive disorders known to exhibit increase risk to cancer such as Fanconi anemia complementation group C (MIM: 227645) and Xeroderma pigmentosum complementation group G (MIM: 278780).

Three of our top candidate variants were predicted to exhibit LoF: *NTHL1* c.244C>T; p.Gln82Ter, *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser. *NTHL1* c.244C>T; p.Gln82Ter has been independently reported in the literature due to its frequency, while *FANCC* c.897G>T; p.Arg299Ser has been reported only in ClinVar database. The introduction of a termination codon in *NTHL1* p.Gln82Ter is predicted to affect NTHL1 protein production, eliciting its classification as PV in ClinVar and by ACMG guidelines. *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser were predicted to affect splicing by all of our selected in silico tools. As RNA was not available from carriers of these variants, we were unable to investigate their effect on the gene transcripts. We applied a stringent criteria for prioritizing missense variants using a selected set of high performance in silico prediction tools [418,420,421,489]. *ERCC5* c.2556A>G; p.Ile852Met and *NEIL1* c.248G>T; p.Gly83Asp, which has been independently reported in the literature due to its frequencies, were among our top prioritized missense variants. A recent study assessed the performance of 44 in silico tools with 70 tool-thresholds combinations in predicting missense variants using a curated dataset of over 9,000 missense variants in five OC and/or BC risk genes that were classified as deleterious or tolerated based on different functional assays [421]. Two of the in silico tools that were selected in our analysis, REVEL with a threshold of >0.7 and VEST with a threshold of ≥0.5 prediction scores of a missense variant being deleterious, were shown to have the best performance of 79% and 74%, respectively [421]. Moreover, a combination of both tools with these prediction score thresholds boosts the prediction performance up to 81% [421]. *NEIL1* c.248G>A; p.Gly83Asp had REVEL and VEST scores of >0.7, while *ERCC5* c.2556A>G; p.Ile852Met had a REVEL score at the threshold of 0.7 and VEST score of 0.9. The biological impact of ERCC5 p.Ile852Met is unknown, though the variant alters a codon in the highly conserved I-Nuclease domain (see **Figure 5.4.**), which may impact ERCC5 endonuclease activity. Whereas, cells expressing NEIL1 p.Gly83Asp have been shown to increase levels of stalled replication forks and double-strand breaks as compared to wild-type NEIL1 [503]. For the missense variants identified in the non-FC cases, eight of the nine missense variants were predicted to be deleterious by REVEL (>0.7) and/or VEST (≥0.5). One variant *NTHL1* c.349C>T; p.Pro117Ser was found to have a REVEL score of 0.6 and

VEST score of 0.5, which is within the intermediate window of prediction scores (REVEL <0.7->0.4; VEST<0.5) where the threshold of predicting missense variants being tolerated is <0.4 [421]. It is evident that in silico tools are being developed with increasing improvement in their performance and are useful alternatives for selecting and prioritizing missense candidates for further characterization [421,514].

We applied stringent criteria to select top candidates for further analyses as it was not feasible to perform WES on all our FC OC study cases. Though selecting for rare variants with MAF ≤0.005 aligns with our hypothesis for identifying candidate moderate-to high-risk variants with the assumption that new candidate genes are transmitted via an autosomal dominant mode of inheritance [99,156,465,485], we filtered out our recently reported, LPV *FANCI* c.1813C>T; p.Leu605Phe [384]. Notable is that the one family harbouring this *FANCI* variant among the 13 familial cases investigated in this study, did not harbour any of our top candidates. As our strategy selected but not eliminated top candidates, further research is required to determine their relevance to OC risk. Moreover, as we have shown in our studies of predicted missense identified in *RAD51C* and *RAD51D* [404] and *FANCI* [384] in OC cases from the FC population, modeling variants by in cellulo assays would provide further evidence for their relevance in OC biology.

Some of our candidate variants or others in these genes that met our selection criteria were identified in 3.3% of 937 non-FC familial or sporadic OC cases of mostly European ancestry 0-0.004% in gnomAD controls, suggesting that our gene candidates may be relevant in other populations. Though this observation was not unexpected, given that approximately 55% of our non-FC OC cases were from the same Australian study group [259], they are also consistent with our analysis of the Pan-Cancer TCGA OC cases. During the course of this study, a recent report investigated a set of DNA repair genes in 33 different cancers from Pan-Cancer TCGA, and they found that missense variants predicted damaging are statistically enriched in OC cases [515]. Moreover, carriers of LoF variants in some of our candidate genes have been reported in HBOC families such as *ERCC5* [516,517]*, FANCC* [518–521] or *NTHL1* [462,505,522] from different populations. Interestingly, LoF variants in *ERCC5* [504], *FANCC* [501] or *NTHL1* [504,505] have been associated with hereditary BC cases in the

context of HBOC families. On the other hand, our literature search did not identify reports of germline PVs in *EXO1* or *NEIL1* in OC, although variants in these genes have been reported in the context of other hereditary cancers such as colorectal cancer [523–525]. However, common variants in *EXO1* have been associated with OC risk [526].

Although we are limited by sample size, we did not observe any striking clinical characteristics regarding age at diagnosis or histopathology of OC disease in carriers of our candidate variants. The average age at diagnosis with OC in FC variant carriers (average=58; median=61 years) is comparable to that of AUS variant carriers as well as PanCancer – TCGA variant carriers (average=59; median=59 years), which in turn is comparable with that of carriers of *BRCA2* PVs (average=58 years) [344] and the general population (median=63 years) [1,3]. The majority of OC cases harbouring the candidate variants had HGSC (84.4%, 38/45), which is the most common subtype of OC reported in epithelial ovarian cancer [20] and thus is overrepresented in our study groups [345]. We did observe three carriers of *EXO1* c.1268-1G>T, *NEIL1* c.248G>A; p.Gly83Asp and *NTHL1* c.244C>T; p.Gln82Ter with mixed histology (serous mixed with endometrioid or clear cell; and endometrioid mixed with mucinous) (see **Table S8**). Interestingly, a survey of our candidate variants in the Ovarian Cancer Association Consortium (OCAC) database (ocac.ccge.medschl.cam.ac.uk/data-projects/, accessed on 15 June 2020), showed that *NEIL1* c.248G>T; p.Gly83Asp was significantly associated with OC overall (odds ratio [OR] = 1.5; p = 0.038), and this association was stronger with the endometrioid subtype (OR = 3.75; p= 0.00008) (see **Table S10**). *NTHL1* c.244C>T; p.Gln82Ter showed a higher OR = 1.5 in clear cell subtype but was not statistically significant (p = 0.36) (see **Table S10**). These observations are interesting as *NTHL1* and *NEIL1* are involved in repairing single stranded-DNA breaks via BER pathway. MMR genes as also involved in repairing single stranded-DNA breaks via MMR pathway that are associated with conferring an increased risk to the endometrioid and clear cell histological subtypes of OC [109]. We could not investigate the other candidate variants *ERCC5* c.2556A>G; p.Ile852Met, *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser from this genotyping-based database as they were not represented in the SNP arrays which could be due to their rarity in the general population.

The role of our candidate genes in the etiology of OC is unknown, though LOH analyses suggest that loss of function of some of our candidate genes in tumour cells may be important in tumourigenesis of OC as has been demonstrated for known OC risk genes [465]. We showed loss of the wild-type alleles in tumours from carriers of *ERCC5* c.2556A>G, *NEIL1* c.248G>T; p.Gly83Asp or *NTHL1* c.244C>T; p.Gln82Ter. We also showed loss of the wild-type alleles in the tumour DNA from the left and the right ovaries from two *NTHL1* c.244C>T carriers with bilateral OC. This suggests the possibility that loss of wild-type alleles occurred at an early stage in tumourigenesis. However, we observed no LOH in four of tumour DNA from one carrier each of *ERCC5* or *NEIL1* variants and two carriers of *NTHL1* variant (see **Table S5.7.**). In previous studies we have also demonstrated complete or partial loss of wild-type alleles in tumour DNA from FC carriers of *RAD51C* c.705G>T; p.Lys235Asn [404], *RAD51D* c.620C>T; p.Ser207Leu [342,404] and *FANCI* c.1813C>T [384] also from the analyses of RRCancer biobank materials. We also observed no LOH in some of the tumour DNA from OC carriers of our *RAD51C* c.705G>T; p.Lys235AsnAs and *RAD51D* c.620C>T; p.Ser207Leu, as the DNA was extracted post-chemotherapy treatment, suggesting the possibility of stromal cells contamination [342,404]. Indeed, all of the DNA tumours from the four cases analysed in this study were confirmed to be extracted after chemotherapy. Interestingly, our LOH analyses in this study provided evidence for loss of variant allele from other candidate variants *EXO1* c.1268-1G>T and *FANCC* c.897G>T carriers. It is not clear if this is due to contaminating normal tissues as our analyses were not performed on selected tumour cells as HGSC samples are often enriched in tumour cells. Srinivasan et al. (2021) recently reported that approximately 20% of the 55 investigated cancers, including OC, showed a retention of the wild-type alleles in the high-penetrant genes such as *BRCA1* and *BRCA2* [154]. A retention of the *RAD51D* c.620C>T; p.Ser207Leu was also observed in tumour DNA from an OC carrier [342]. It is not clear if such cases reflect a reversion of variant to wild type allele as has been shown with *BRCA1* or *BRCA2* carriers in the context of developing resistance to cisplatin or the targeted therapy poly (ADP-ribose) polymerase (PARP) inhibitors [527,528]. Further research is required at the tumour cell level to determine biological impact of variants in the context of wild-type alleles in carriers.

5.7. Conclusion

Our WES and genetic analyses of 468 genes directly or associated with DNA repair pathways in study groups from a genetically defined population identified candidate variants in *ERCC5*, *EXO1*, *FANCC*, *NEIL1* or *NTHL1*. The genetic analyses of these variants and genes in non-FC OC study groups implicate these genes in other populations. Genetic epidemiology of variant carriers and functional assays to assess the biological impact of variant proteins could elucidate the effect of candidate variants to OC risk.

5.8. Supplementary Materials

See **Appendix VIII**.

5.9. Acknowledgment

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5.10. Conflict of interest

The authors declare no competing interests.

5.11. Ethical approval

This project was conducted and approved according to the guidelines of The McGill University Health Centre Research Ethic Board (MP-37-2019-4783) (see **Appendix IX**).

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CHAPTER VI: General discussion

6.1. General summary and discussion

I used WES and bioinformatics analyses to pursue my proposed strategies for investigating germline PVs in highly selected OC families and/or cases from the FC population of Quebec, a defined population exhibiting founder effect and genetic drift. I focused on genes involved in DNA repair pathways in order to identify: (1) likely PVs in known OC risk genes, one in *BRCA1* (Chapter II) [417], five in *RAD51C* or *RAD51D* (Chapter III) [404] and three in *BRIP1* (Chapter IV); and (2) five potentially deleterious candidate variants in new genes that previously have never been implicated in OC predisposition, *ERCC5*, *EXO1*, *FANCC*, *NEIL1,* and *NTHL1* (Chapter V) that may also be relevant to non-FC populations. The overall frequency of carriers of each of these variants is small and collectively accounts for approximately 15% of the investigated OC families, each with at least two or more close relatives with OC; 6% of the OC cases were not selected for age at diagnosis or for family history of cancer; and less than 1% of the population-matched controls. The overall observed carrier frequencies are consistent with the expected trend of a higher frequency of variant carriers in familial cancer cases than in sporadic cancer cases and then those in the cancer-free controls; this has been observed in known CPGs [165,384]. My customized filtering and prioritizing criteria allowed me to focus on the role of the type of variant in the context of predisposition to OC, which has been demonstrated to have a potential impact on gene biological function at the level of the RNA transcript or protein (Chapters II to V), supporting the potential pathogenicity and clinical implications of the variants in the context of hereditary OC. Altogether, my approach and genetic analyses of PVs in the germline of women with OC negative for *BRCA1* or *BRCA2* PVs support my hypothesis that there are other PVs in known or new candidate OC risk genes involved in DNA repair pathways.

Investigating the germline of families with at least two close relatives with OC allowed me to characterize new candidate variants in the known OC risk genes, *RAD51C*, *RAD51D* (Chapter III) [404] and *BRIP1* (Chapter IV) as well as to identify new candidate variants in DNA repair genes that previously had not been implicated in OC risk (Chapter V). The likelihood of identifying PVs that are associated with the disease is

increased in these families in contrast to cases not selected for family history of cancer [182]. The trend of the overall carrier frequencies for these potentially deleterious variants was considerably higher (15.4%) in 52 familial OC cases than that in the 435 sporadic OC cases (5.5%) and in 1025 cancer-free controls (0.3%). These findings are in agreement with the trend that has been observed for several decades in known CPGs [165,182], and further supports the candidacy of the potentially deleterious variants (Chapters II to V). For comparison, the trend for PVs in *BRCA1* and *BRCA2* combined was 53.8% in 52 familial OC cases versus 9.4% of 435 sporadic OC cases. I was restricted in assessing the carrier frequency of *BRCA1* and *BRCA2* PVs in the 1025 cancer-free controls because we did not have ethics approval to test these variants. However, the frequency of the most frequently occurring PVs in *BRCA1* and *BRCA2* in the FC population of Quebec was recently estimated to be 0.1% in 1937 population-matched, cancer-free controls [381]. Indeed, applying this strategy, in accordance with my proposed strategy I, facilitated the identification of potentially deleterious variants implicated in OC predisposition.

Applying a candidate gene approach facilitated the identification of potentially deleterious candidates that are involved in DNA repair pathways implicated in predisposition to OC. Genes encoding proteins that directly interact with known OCPGs like *BRCA1* and *BRCA2* or those involved in the same pathway may confer risk resulting in the same phenotype [151,179,182]. Collectively for *BRCA1* and *BRCA2* PVs, 69.1% of the 52 OC families and 15.3% of the 435 sporadic OC cases, are accounted for by a potentially deleterious variant in a DNA repair pathway. These contributions are generally consistent with other reports [68,100,151,259]. For the OC families, the candidate variants that I discovered did not account for all of the remaining OC cases negative for *BRCA1* and *BRCA2* PVs which can be attributed to the genetic heterogeneity in OC as expected. Other types of genetic events in known OC risk genes may account for one or two of these families, but such events like intronic PVs (as I showed in Chapter II) or germline copy number variation are expected to be rare [229,529,530]. My analysis was based on a curated list of 486 genes directly or indirectly involved in DNA repair according to our current knowledge. I cannot exclude the possibility that the remaining familial OC cases negative for a potentially deleterious

candidate variant may harbour PVs in other DNA repair genes that were not part of my extensive list [151,531,532]. Another possibility is that these families may harbour a PV in genes that are not involved in the DNA repair pathway [68,259]. It is also possible that the clustering of OC cases in at least one or two of the families occurred simply by chance [297], given that the estimated heritability of OC is approximately 39% (95% CI: 23 to 55) based on a twin-based study [102]. Applying a candidate gene approach, in accordance with my proposed strategy II, allowed me to identify potentially deleterious variants as candidates involved in various DNA repair pathways in OC families and cases.

The unique genetic architecture of the FC population allowed me to identify multiple OC carriers of rare, potentially deleterious candidate variants in known and new candidate OCPGs. The likelihood of identifying rare PVs is increased for those that frequently occurring in the FC population [182,319,320] The overall frequency of carriers of each variant is small, relative to those in *BRCA1* and *BRCA2* [182], and collectively they account for 15.4% of 52 familial OC cases and 5.5% of 435 sporadic OC cases compared to 0.3% of 1025 population-matched controls. The frequently occurring *RAD51C* c.620C>T; p.Ser207Leu variant in the general population only accounts for 5.8% of the 52 OC families and 3.4% of the 435 sporadic OC cases compared to 0.1% of the population-matched controls [342,404]. The carrier frequencies of the other recurring variants were lower compared to the *RAD51D* variant: *BRCA1* c.5407-25T>A (Chapter II) [417], *RAD51C* c.705G>T; p.Lys235Asn and c.414G>C; p.Leu138Phe, *RAD51D* c.137C>G; p.Ser46Cys (Chapter III) [404], *BRIP1* c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu and c.2990_2993delCAAA; p.Thr997ArgfsTer61 (Chapter IV), *ERCC5* p.Ile852Met; c.2556A>G, *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser (Chapter V). The remaining candidate variants, *NEIL1* c.248G>T; p.Gly83Asp and *NTHL1* c.244C>T; p.Gln82Ter, were identified in multiple OC cases due to their higher frequency in the general population compared to the other candidate variants. Nevertheless, the overall carrier frequencies of the identified candidate variants generally align with the previously reported frequencies of likely PVs in other DNA repair genes such as *BRCA1* and *BRCA2* [100,151]. In addition to the fact that the overall frequencies of candidate variant carriers are small, the variation in the carrier frequency

of these candidate variants is consistent with genetic drift due to the waves of localized expansion of the FC population that occurred in Quebec since the founding of this European population in 1608 [182,319,320,322]. The dynamics of the changes in the genetic architecture of the FC population over time have been proposed to account for the varying frequencies of carriers of up to 40 different PVs in *BRCA1* and *BRCA2* in this population [182]. We cannot exclude the possibility that cases that harbour the same variant are closely related and inherited a copy of the variant from a common ancestor as genealogical information was not available for all the OC cases investigated in my studies as was shown by Dr. Tonin's group and others [182]. Altogether, applying my proposed strategy III allowed me to identify potentially deleterious recurring variants implicated in OC cases in the FC population of Quebec that may also be relevant to non-FC populations.

The filtering and prioritizing criteria that I used allowed me to focus on the role of likely missense PVs in known and new candidate OCPGs. Although I applied relatively relaxed filtering criteria for variants with MAF ≤0.01 in *RAD51C*, *RAD51D* (Chapter III) and *BRIP1* (Chapter IV), genes known to be implicated in OC risk, the likely PVs that I identified were found to have a MAF ≤0.005 which is consistent with known PVs in these genes as well as in *BRCA1* and *BRCA2* [33,156,168,241,465,533]. In fact, the findings of rare variants with a MAF ≤0.005 aligns with my hypothesis for identifying moderate-to-high risk candidate variants under an autosomal dominant mode of inheritance and is consistent with the prevalence of OC [299,485]. As a result, I used a MAF ≤0.005 cut-off for selecting variants in other DNA repair genes (Chapter V). While using a stringent MAF cut-off to identify likely PVs, the loosening of other restrictions and the use of high performance in silico tools enabled me to capture a number of variants that are usually filtered out from typical analyses for identifying PVs. In general, the majority of studies focusing on identifying cancer risk variants restrict their analyses to LoF variants which includes nonsense, frameshift and canonical splicing variants [534]. This can be justified by the fact that LoF variants are predicted to affect protein production due to premature amino acid termination eliciting nonsense-mediated mRNA decay [535] and thus their classification as PVs. Studies that have been conducted to identify new OCPGs are summarized in **Table 6.1.** In the original research studies, I identified a total of:

Table 6.1. Description of published studies focused on identifying new ovarian cancer predisposing genes.

Year	Type of study (Number of phases)	Number and type of OC cases (*BRCA1* and *BRCA2* status)	Ethnicity or geography of cases	Number and type of controls	Type of analysis for cases	Number of analyzed genes	Proposed candidates involved in DNA repair pathways	Reference
2021	Family-based and case-control (Three phases)	1 OC family (negative) 42 OC families (regardless) 82 HBOC families (regardless) 158 HBC families (regardless) 439 Sporadic OC cases (regardless) 558 Sporadic BC cases (regardless) 516 Sporadic Australian OC cases (negative) 412 Sporadic TCGA-OC cases (regardless)	French Canadian	2950 population-matched cancer-free females	WES, targeted sequencing of coding and splicing regions and SNP genotyping – all captured variants with MAF ≤0.01, and targeted genotyping	276 DNA repair genes	*FANCI*	[384]
2021	Case-control (Two phases)	6385 OC cases (regardless) 141135 OC cases (regardless)	European	18930 and 9685 population-matched controls	Same as above	54 DNA repair genes	*PALB2*	[255]
2020	Family-based (One phase)	14135 OC cases (regardless) 11 OC families	Brazil	Population-matched	WES and targeted sequencing of coding	2319 genes	*DROSHA, FAN1,*	[536]

				AbraOM controls	and splicing regions – all captured variants with MAF<0.01		*POLQ and RAD54L*	
2020	Case-control (One phases)	516 OC cases (negative)	Australia	Non-cancer, non-Finnish European gnomAD population-based controls	WES only – all captured LoF variants with MAF ≤0.005	All captured genes	*MRE11A, RAD1* and others	[259]
2020	Family-based and case-control (Two phases)	140 OC families (negative) 381 Sporadic TCGA-OC cases (regardless)	United States	ExAC controls	WES only – all captured LoF variants with MAF ≤0.001	All captured genes	*POLE*	[474]
2019	Case-control (One phase)	2051 Sporadic OC cases	United States	In-house population-matched controls[1] and gnomAD controls	WES only – all captured variants with MAF ≤0.005[2]	625 cancer-associated genes	*ATM, CHEK2 and PALB2*	[260]
2017	Family-based (One phase)	48 OC families or early-onset cases (negative)	United States	Not applicable	WES and targeted sequencing of coding and splicing regions – all captured LoF	217 DNA repair and cell cycle genes	*ATM, CHEK1, FANCM, HMMR, MCM4,*	[537]

					variants with MAF<0.01		*PALB2, POLK, POLQ, RAD1, RAD52, REC8 and TP53I3*	
2017	Case-control (Three phases)	412 Sporadic TCGA-OC cases (regardless) 3107 Sporadic HGSC cases (regardless) 1491 Sporadic OC cases (regardless) 1491 Sporadic OC cases (regardless)	European	3368 population-matched controls	Same as above	12 DNA repair genes[3]	*FANCM*	[473]
2014	Case-control (One phase)	429 Sporadic TCGA-OC cases (regardless)	Various	557 cancer-free the Women's Health Initiative Exome Sequencing Project (WHISP) females	WES only – all captured LoF variants with MAF <0.01	All captured genes	*PALB2* and *FANCM*	[68]

[1] The number of controls varies among the analyzed genes; [2] Loss-of-function (LoF) variants were excluded if the predicted premature termination was located beyond the last 55 base pairs; [3] Gene selection based on enrichment analysis of The Cancer Genome Atlas (TCGA) -Ovarian cancer (OC) cases and controls. ExAc: The Aggregation Exome Database; gnomAD: The Aggregation Genome Database; MAF: Minor Allele Frequency; and WES: Whole Exome Sequencing.

(1) two nonsense variants *RAD51D* c.694C>T; p.Arg232Ter (Chapter III) and *NTHL1* c.244C>T; p.Gln82Ter (Chapter V); and (2) one frameshift variant, *BRIP1* c.2990_2993delCAAA; p.Thr997ArgfsTer61 (Chapter IV), out of five identified candidate variants. Due to the rarity of such variants in OC families and cases, I expanded my search to all types of variants using a selected set of high performance in silico tools for predicting variants that affect splicing or affect the biological function of the encoded protein [418,420,421,489]. For variants predicted to affect splicing, the most commonly used in silico tools predict variants that are located at intronic splicing junctions [538]. Newly developed tools such as SpliceAI [427] have been designed to predict variants located beyond splicing regions or those located within coding regions to create new splice sites [417]. Hence, although these variants are typically filtered out for further analyses, my customized WES analyses allowed me to capture these variants in known and new candidate OCPGs. Indeed, I was able to identify different types of variants affecting splicing in addition to the canonical splicing variant, *EXO1* c.1268-1G>T (Chapter V). For example, I identified: (1) an intronic variant in *BRCA1* c.5407-25T>A (Chapter II); and (2) two exonic missense variants that were predicted to affect splicing, *RAD51C* c.705G>T; p.Lys235Asn (Chapter III) and *FANCC* c.897G>T; p.Arg299Ser (Chapter V). The predicted impact on splicing by some of these variants was supported by different functional assays [404,417,539,540]. For missense variants, I relaxed the criteria for prioritizing missense variants in *RAD51C*, *RAD51D* (Chapter III) and *BRIP1* (Chapter IV), such that a PV was predicted by at least one out of the selected tools, given the known role of these genes in OC predisposition. In total, I selected seven missense variants in *RAD51C*, *RAD51D* or *BRIP1*. Five of the seven missense variants were predicted to be deleterious by six of the selected tools, including two tools (REVEL with a threshold of >0.7; and VEST with a threshold ≥0.5 for the prediction of a missense variant being deleterious) that both tools were recently shown to have the best performance of 79% and 74% [421]. The performance of REVEL and VEST among 42 other in silico tools with 70 tool-threshold combinations for predicting missense variants was assessed using a curated dataset of over 9,000 missense variants in five OC and/or BC risk genes that were classified as deleterious or tolerated based on different functional assays [421]. Indeed, these five variants were found to affect the

biological function of the encoded protein by in cellulo assays: *RAD51C* c.414G>C; p.Leu138Phe [231], *RAD51D* c.137C>G; p.Ser46Cys and c.620C>T; p.Ser207Leu [342,404] and *BRIP1* c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu, but not *BRIP1* c.415T>G; p.Ser139Ala and c.1216G>T; p.Ala406Ser. Based on my experience gained from the analyses in Chapters III and IV and before the publication of Cubuk et al. in 2021 [421], I used this stringent criteria to identify new potentially deleterious candidate variants for OC risk (Chapter V) by prioritizing missense variants predicted to be deleterious by at least six out of eight in silico tools. I identified *ERCC5* c.2556A>G; p.Ile852Met and *NEIL1* c.248G>T; p.Gly83Asp which had REVEL and VEST scores of ≥0.7. Although the biological impact of ERCC5 p.Ile852Met; c.2556A>G is not known, it was shown that cells expressing NEIL1 p.Gly83Asp have increased levels of stalled replication forks and double-strand breaks as compared to cells expressing wild-type NEIL1 [503]. Altogether, it is evident that the performance of in silico prediction tools is continually improving and that they are becoming more and more useful as alternatives for selecting and prioritizing missense candidates for further characterization [421,514].

The role of my candidate variants in the etiology of OC is not known, though LOH analyses would suggest that some of these candidates may be crucial to ovarian tumourigenesis [465]. As has been demonstrated for known OC risk genes for many decades, I observed complete or partial loss of the wild-type allele in tumour DNA from: (1) one carrier of the likely intronic PV in *BRCA1* (Chapter II); (2) 12/15 (80%) carriers of the likely PVs in *RAD51C* or *RAD51D* [404] (Chapter III); (3) 1/4 (25%) carriers of the likely PV in *BRIP1* (Chapter IV); and (4) 5/11 (46%) carriers of the potentially deleterious candidate variants in *ERCC5*, *NEIL1* and *NTHL1* (Chapter V). These analyses provide additional evidence that the known OC risk genes, *RAD51C*, *RAD51D* and *BRIP1*, as well as the new candidate genes may behave as tumour suppressors, similar to the established OC risk genes *BRCA1* and *BRCA2* [154,465,541]. Interestingly, I observed loss of the wild-type allele in tumour DNA from the left and the right ovaries of two carriers of the *RAD51D* c.620C>T; p.Ser207Leu variant [404] and two of the *NTHL1* c.244C>T; p.Gln82Ter variant, suggesting that such events occur at an early stage in tumourigenesis [542]. Other tumours, however, had no observed LOH: (1) 3/15 (20%) carriers of the likely PVs in *RAD51C* or *RAD51D* [404]; (2) 2/4 (50%)

carriers of the potentially tolerated variants in *BRIP1*; and (3) 4/11 (36%) carriers of the potentially deleterious candidate variants in *NEIL1* or *NTHL1*. With the exception of one case, DNA from these carriers was extracted post-chemotherapy treatment, suggesting the possibility of contamination with stromal cells [342,404,541]. Indeed, partial or complete loss of the wild-type allele was observed in DNA extracted mostly from tumour tissues of the FFPE sections. However, it has been recently reported that approximately 20% of 55 investigated cancers, including OC, showed heterozygosity in tumours harbouring germline PVs in highly penetrant genes such as *BRCA1* and *BRCA2* in contrast to 40-60% of the tumours harbouring germline PVs in the low-to-moderate risk genes [154]. In the original research studies, I investigated only one inactivation mechanism involving the loss of the wild-type allele in the tumour DNA from carriers of candidate variants. LOH of the wild-type allele has been shown over the years to be the predominant inactivation mechanism of *BRCA1* and *BRCA2* [68,154,165,465,543,544]. However, there are other mechanisms that merit further investigation such as acquiring a somatic in-trans PV, which is different from the germline PV, or gene silencing through promoter methylation of the in-trans allele [165,465]. For example, evidence is accumulating to support the idea that methylation of *RAD51C* is another mechanism of inactivation, as it is for *BRCA1* [545–547]. Another possible inactivation mechanism is that the candidate CPG is haploinsufficient, meaning that losing both alleles is not required to confer cancer risk; in other words, losing one allele despite retaining a functional in-trans copy is sufficient to increase the risk for developing the cancer [165]. Heterozygous carriers of the *NTHL1* c.244C>T; p.Gln82Ter variant have been recently reported to be at increased risk for developing BC, and that the risk was proposed to be through haploinsufficiency [505]. In my case (Chapter V), the *NTHL1* locus is located in chromosome region 16p13.3, which is one of the most frequently regions lost in OC [30]. This is in agreement with my observations of the loss of the wild-type allele in two of *NTHL1* c.244C>T; p.Gln82Ter OC carriers. However, I had very few OC carriers of this *NTHL1* variant to confirm this possible inactivation mechanism in the context of OC. More OC carriers of PVs in *NTHL1* are required to address this question. In the remaining analyses of LOH, interestingly, I observed retention of the wild-type allele in two out of the 11 carriers of the *EXO1* c.1268-1G>T and *FANCC* c.897G>T;

p.Arg299Ser variants. It is not clear if such cases reflect a reversion of the variant allele to the normal allele as has been shown with *BRCA1* or *BRCA2* carriers in the context of developing resistance to the OC standard-of-care chemotherapy treatment such as cisplatin or the targeted PARP inhibitor therapy [87,527,528]. A retention of the *RAD51D* c.620C>T; p.Ser207Leu variant was also observed in tumour DNA from one of the OC carriers [342]. Further research is required to determine the biological impact and treatment response of these variants.

The clinical implications of PVs in genes involved in DNA repair and the response to cisplatin, carboplatin or PARP inhibitors is an active area of research and clinical trials [401,548,549]. OC cases with tumours that are deficient for BRCA1 or BRCA2 are now known to be particularly sensitive to chemotherapy as well as to PARP inhibitors due to the deficiencies in the HR pathway via synthetic lethality [94,549–556]. In Chapters III and IV, it was demonstrated that cells expressing the RAD51D c.137C>G; p.Ser46Cys [404] variant or either of the variants in BRIP1 c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu or c.2990_2993del; p.Thr997ArgfsTer61 confer cellular sensitivity to the DNA inter- or intra-strand crosslink-based agents, cisplatin and/or mitomycin C (MMC), as expected [202,203,342,557]. This phenotype was consistent with two other variants I identified as part of Chapter II, RAD51C c.414G>C; p.Leu138Phe [231] and RAD51D c.620C>T; p.Ser207Leu [342] which were independently reported to mediate cellular sensitivity to these agents. Although such assays have not been performed specifically for carriers of the RAD51C c.705G>T; p.Lys235Asn and RAD51D c.694C>T; p.Arg232Ter as LoF variants, RAD51C [558–560] or RAD51D [342,404] knock-out cells have been shown to confer sensitivity to cisplatin, carboplatin and/or MMC. In addition to conferring sensitivity to these DNA inter- and intra-strand crosslink-based agents, RAD51D c.137C>G; p.Ser46Cys [404] was found, as expected, to lead to synthetic lethality and subsequently confer cellular sensitivity to the targeted-based PARP inhibitors, olaparib and talazoparib [91]. This is in contrast to the likely PVs BRIP1 c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu or c.2990_2993del; p.Thr997ArgfsTer61, suggesting that the associated risk of harbouring such variants in *BRIP1* is HR pathway-independent [455]. While the role of the five potentially deleterious candidate variants in other DNA repair genes

(Chapter V) in the response to chemotherapy or PARP inhibitors is not known, sensitivity to cisplatin is speculated for tumours deficient for EXO1 [510,561] and FANCC [562–564] as they are part of the HR and FA pathways, respectively. Unlike EXO1 [565,566], however, FANCC deficiency may not confer sensitivity to PARP inhibitors given that the other protein-coding genes involved in the FA pathway, including *BRIP1*/*FANCJ* (Chapter IV), do not confer sensitivity [384,455,457]. Interestingly, *ERCC5* has been proposed as a favorable prognostic candidate marker for OC response to chemotherapy; a correlation between the loss of chromosome region 13q and the downregulation of *ERCC5* was observed to be associated with prolonged progression-free survival of OC cases [567]. This phenotype is consistent with our current knowledge of protein-coding genes involved in the nucleotide excision repair (NER) pathway, including *ERCC5* [502], that can recognize and remove cisplatin-induced DNA damage [568–570]. However, it remains controversial whether deficiency of any of the protein-coding genes involved in the NER pathway, including *ERCC5*, would lead to synthetic lethality and subsequently confer sensitivity to PARP inhibitors [569,571–574]. The role of *NTHL1* and *NEIL1* in cisplatin and PARP inhibitor sensitivity is generally not known based on the current knowledge of protein-coding genes involved in the NER pathway [574,575]. It was demonstrated that knockout cell lines for specific protein-coding genes involved in the base excision repair (BER) pathway confer sensitivity to cisplatin and PARP inhibitors [576,577], but none were in the *NTHL1* or *NEIL1* genes. Taken altogether, the majority of protein-coding genes involved in DNA repair pathways confer sensitivity to OC standard-of-care chemotherapy treatments, yet only a select few of these genes confer sensitivity to PARP inhibitors, which could be driven via a HR deficiency.

The consequence of harbouring PVs in two or more autosomal dominantly inherited CPGs is known. This phenomenon is called multi-locus inherited neoplasia allele syndrome (MINAS) [578–580]. Cases with MINAS are available online (databases.lovd.nl/shared/diseases/04296). The prevalence of such multi-locus genetic events, the associated phenotype and the clinical implications, however, remain unknown. The associated phenotypes of individuals with MINAS are hypothesized to be: (1) additive when harbouring PVs in more than one CPG would result in the

occurrence of multiple primary tumours each specific to the affected gene; or (2) synergistic when harbouring PVs in a combination of CPGs would result in a more severe phenotype such as an earlier age of onset or an occurrence of an atypical aggressive tumour [578,580]. The prevalence of MINAS is predicted to be extremely rare, affecting approximately 1 in 190,000 individuals in the Western European population [289], yet this is likely underestimated given new evidence from the rapidly increasing number of gene-panel sequencing, WES or whole genome sequencing (WGS) of cancer cases [578,579,581]. The prevalence of MINAS is expected to be even higher in populations exhibiting a unique genetic architecture due to genetic drift [578,579]. For example, the prevalence of MINAS is estimated to be 1 in 1,800 individuals in the Ashkenazi Jewish population [289], yet it is not known in the other well-recognized founder populations, including the FC population of Quebec. A recent systematic literature review reported that of 385 cancer cases with PVs variants in two or more of 94 known CPGs, about 34% harboured PVs in *BRCA1* and *BRCA2* [578], three of which were OC cases of FC descent [345,582]. During WES analyses of the candidate variant carriers in the original research studies on known OC risk genes (Chapters III and IV), I identified two out of 22 FC OC cases that harboured PVs in two known OC risk genes. One case harbouring the *RAD51D* c.694C>T; p.Arg232Ter variant was also found to harbour *BRCA1* c.1462dupA; p.Thr488AsnfsTer2, which has not been previously reported in the FC population [404]. The other one harboured the *BRIP1* c.2990_2993del; p.Thr997ArgfsTer61 variant which was found to also harbour *BRCA1* c.962G>A; p.Trp321Ter, which is one of the frequently occurring rare variants in the FC population [182,344,347,583]. Interestingly, cancer carriers of either of these two variants and PVs in other known risk genes have been reported [580,584]. In Chapter V, I also identified the *NEIL1* c.248G>A; p.Gly83Asp variant in one of the 14 analyzed OC cases that also harbours a PV in *BRCA1* c.5102_5103del; p.Leu1701GlnfsTer14, which is one of the frequently occurring rare variants in the FC population [182,344,377,507]. In the recent report by McGuigan et al., (2021) it was reported that 28% (108/385) of MINAS cases harbouring PVs in *BRCA1* and *BRCA2* developed multiple primary tumours, of which 31% (33/108) developed more than two primary tumours [578], whereas, 6.5% (25/385) of the MINAS cases harbouring PVs in other

CPGs including *BRCA1* or *BRCA2* developed atypical tumours associated with the affected genes [578,579]. No phenotype such as younger age at diagnosis or developing other primary tumours was observed in the abovementioned OC carriers of two PVs.

6.2. Limitations

There are several limitations to my original research designs and biases in the selection of the cases and controls that should be acknowledged. Future directions to overcome some of these limitations are provided in **sub-section 6.3**.

One of the general limitations of my studies (Chapters II to V) was that I was not able to estimate the associated OC risk of any of the candidate variants to support their candidacy as OC predisposing variants [299] because I was limited in the sample size of the cancer cases and controls. Typically, risk association studies require sample sizes in the range of thousands [240,241,255,505], while I only had a few hundred cases. In an attempt to overcome this limitation, I retrieved the associated ORs for selected candidates from the genotyping-based OCAC database, which includes *RAD51D* c.620C>T; p.Ser207Leu (OR_{log2}: 17.2 with HGSC OC subtype; P=0.00001) [182] (Chapter III) [404], *NEIL1* c.248G>A; p.Gly83Asp (OR_{log2}: 1.3 with EC OC subtype; P=0.00008) and *NTHL1* c.244C>T; p.Gln82Ter (OR_{log2}: 0.41 with CC OC subtype; P=0.363) (Chapter V). In comparison the OR of the most frequently occurring PV in *BRCA1* (c.4327C>T; p.Arg1443Ter) in the FC population [182] is (OR_{log2}: 1.2 with HGSC OC subtype; P= 0.00905) [404]. The associated OC risk of one of the identified PVs in *RAD51D* (c.694C>T; p.Arg232Ter) (Chapter III) [404], which is one of the most frequently occurring variants in European population, has been estimated (OR: 16.07; 95% CI: 5.12 to 50.46; P<0.0001) [238,240]. These ORs provide some insight into the associated risk of these variants with OC in general and with specific histopathological OC subtypes, which is informative for determining the required sample size of cases and controls for assessing OC risk.

In addition to providing some insight into the associated risk of some of the candidate variants, another important element of my study was being able to investigate FC OC families and cases that are negative for PVs in known OC risk genes including

BRCA1 and *BRCA1.* Indeed, as mentioned above, I was able to successfully confirm that all of the FC OC families and cases are negative through WES analysis [417]. One of the limitations of WES to identify PVs, however, is that it does not allow one to investigate the possibility of the occurrence of large germline CNVs in *BRCA1* or *BRCA2*. Such events, however, are unlikely for two main reasons. First, the majority of the cases in this study were tested by gene-panel testing which usually includes the analysis of *BRCA1* and *BRCA2* germline CNVs, confirming that these OC cases were negative for CNVs in both genes. Second, the analyses for *BRCA1* and *BRCA2* germline CNVs in FC HBOC and HBC families using the multiplex ligation-dependent probe amplification (MLPA) assay revealed no occurrence of large germline CNVs, suggesting that such an event is rare in the FC population [585]. To date, the contribution of large germline CNVs in the other known OC risk genes such as *RAD51C* [229] is still not known in the FC population, yet it is expected to be rare. Indeed, large CNVs in BC risk genes including *BRCA1* and *BRCA2* account for about 1% of PVs in over 80,000 BC cases, of which the majority are of Western Europeans origin [586]. This rate of large germline CNVs is consistent with the previously reported estimated rate of 1.8% in 17 known OC and/or BC risk genes in over 4,000 OC or BC cases from HBOC families using in silico prediction tools [229]. Overall, while the technology used for certain analyses may have had certain limitations, I was able to use alternative and complementary methods alongside evidence from the literature to ensure that any shortcomings due to the approach that I used were limited.

While technological limitations were challenging, another important barrier in my studies was a lack of biological material that was needed to better characterize some of my findings. Specifically, there was limited RNA material available from OC cases to confirm the predicted effect of splicing of two of the candidate variants: *BRCA1* c.5407-25T>A (Chapter II) [417], *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser (Chapter V), but not for *RAD51C* c.705G>T; p.Lys235Asn (Chapter III) [404]. This was due to the fact that only DNA from PBL samples were bio-banked for most of the OC cases. Likewise, I was not able to perform LOH analyses on all OC carriers of the candidate variants as extracted DNA from fresh frozen tumours or histopathological blocks for DNA extraction for some of these carriers were not available from the

respective biobanks. A lack of stored biological material from the biobanks and the fact that some of the families date back to the early 2000s explains why genotyping PBL DNA of other family members to further support the candidacy of my variants [299,416] was not feasible.

One of the specific limitations regarding Chapter V that should be noted was rather than considering all possible genes in my analysis, I used a candidate gene approach by investigating genes involved in DNA repair pathways, although the PBL DNA of the OC families was subjected to WES. Using this approach can be justified by the following: (1) DNA damage and genomic instability due to deficiencies in the DNA repair machinery is one of the hallmarks of cancer [70]; (2) this strategy has been shown to successfully identify candidate genes that are now known to be implicated in several hereditary cancers [66,469,587–593] including OC [259,474]; (3) all known and associated OCPGs are involved in DNA repair pathways in contrast to BC [151,156,182,594]; and moreover (4) tumours deficient in some of the genes involved in DNA repair pathways have specific treatment responses in the clinic [527,548,575,595]. In my analyses, I was limited by the sample size required to perform WES combined with the extensive statistical analyses needed to identify candidate variants or genes [259,474]. However, I performed global analyses of WES data generated from all of the OC cases with a PV to look for any co-occurring PV in known OC risk genes [101] or in other genes reported to be associated with different cancers [407]. One of the 16 FC carriers were found to also carry potentially deleterious variants in these genes. Hence, while the sample size in my study was limited, preventing me from analyzing all the genes in the exome data for candidate variants, I was able to make the most of the data available to derive interesting and potentially clinical useful results. In addition, there is a lack of in cellulo assays in Chapter V to support the prediction of candidate variants, although I hoped to have gathered sufficient evidence to merit further future undertakings of the variants that I identified. Already one of the candidate variants, *NEIL1* c.248G>A; p.Gly83Asp, has been shown to have a potentially damaging effect [503] and it illustrates that the strategies that I used in this work are successful in identifying clinically relevant variants.

There are different types of biases that should be acknowledged in the ascertainment of the OC families and cases as well as the controls that were investigated in my original research studies. One of the expected biases is an ethnicity reporting bias. Ethnicity is defined as membership in a group with common national or cultural traditions [596,597]. All the OC index cases from the families that were recruited self-reported as FC of Quebec, of which more than 70% of these cases reported that all four grandparents were FC [344,347,352], and at least 88% of the sporadic cases self-reported as FC of Quebec [345,404]. Only those self-reporting as FC of Quebec were selected for our population-matched controls [404]. Such self-reported ethnicity would affect the identification of candidate variants and the determination of their carrier frequencies due to admixture [598]. Despite not performing any genomic ancestral analyses on the OC cases and controls to support that they are all of FC of origin [597], I identified some recurring rare PVs in the general population (MAF<0.0001): *RAD51C* c.705G>T; p.Lys235Asn (Chapter III) [404], *BRIP1* c.797C>T; p.Thr266Met, c.2087C>T; p.Pro696Leu and c.2990_2993delCAAA; p.Thr997ArgfsTer6 (Chapter IV) and *ERCC5* p.Ile852Met; c.2556A>G (Chapter V). It is plausible that the OC cases harbouring these variants share common ancestors given the history of the FC population in Quebec. Another bias in my studies is that more than half the OC families where the confirmed index case reported another close relative with OC, did not have confirmed pathology reports or death certificates to support the diagnosis. Nevertheless, it has been demonstrated that the probability of agreement between the reported first-degree relatives with OC and the confirmed OC status by pathology report is reasonably accurate 83.3% (95% CI: 72.8% to 93.8%) compared to other gynecological cancers, yet these probabilities decrease significantly with the reporting of second- and third-degree relatives with cancer, including OC [599]. The unconfirmed OC cases in the majority of the OC families were either first- or second-degree relatives to the confirmed index OC case, and only one was a third-degree relative. This suggests that the error arising from this reporting bias should be minimal.

Finally, the last type of bias existing in the OC families and cases is selection bias. The majority of my OC families and cases were recruited from the region of Montreal, while the controls were recruited from different regions of Quebec. This would affect the

frequency of the candidate variants as it is well documented that there has been regional genetic drift in the FC population of Quebec [319,320,327,334,347,600].

6.3. Future direction

There are several pieces of evidence at the level of the variant or the gene that would collectively support the candidacy of the variants and/or genes I identified in my original research studies that are the basis of this thesis in OC risk [299].

Since I started my Ph.D. studies in 2016, the classification of *RAD51C*, *RAD51D* and *BRIP1* as OC risk genes has been consistently supported, yet the level of evidence-based medicine for clinical management has not reached the same level as that for *BRCA1* and *BRCA2* [99,101] (cebm.ox.ac.uk). Therefore, not much benefit might be gained by assessing the associated risk of the likely PVs I identified in these genes (Chapter III-IV). However, I think that it is critical to support the candidacy of the potentially deleterious variants I identified in the DNA repair genes, *EXO1*, *ERCC5*, *FANCC*, *NEIL1* and *NTHL1*, as these have not yet been implicated in OC risk (Chapter V). Specifically, it would be important to assess the associated risk of each of the specific variants that I identified [299] in: (1) large OC families positive for these variants [243,285] and/or (2) large population-based OC cases and population-matched controls [33,255]. One of the barriers to overcome in such an undertaking, however, is that it is expected that OC families positive for some of these variants may be rare, and it would take an international effort to collect a sufficient number of families to be able to assess the associated risk. Alternately, the associated risk could be assessed by the gene-based statistical analysis of LoF and/or potentially deleterious missense variants in OC cases compared to population-matched controls [299,300,510,601,602]. Also, some of these variants could be associated with the other histopathological subtypes of OC such as is the case for *NEIL1* and *NTHL1*; therefore, assessing their associated risks should be carefully addressed.

Understanding the biological impact of the identified candidate variants and the relevant cellular response to the OC standard-of-care chemotherapy treatment, as well as the targeted therapy mentioned in this thesis, would support the prediction of a potentially deleterious variant. Among the identified candidates, four different types of

alternative splicing were predicted: an intronic variant in *BRCA1* (Chapter II) [417], exonic splicing variants in *RAD51C* (Chapter III) [404] and *FANCC* (Chapter V) and a canonical splicing variant in *EXO1* (Chapter V). I was able to show the aberrant splicing of the *RAD51C* c.705G>T; p.Lys235Asn transcript which was confirmed by another independent group [540]. During the course of my investigation of the *BRCA1* c.5407-25T>A variant, the impact of this variant on the gene transcript was also confirmed by an independent group [539]. The impact of the remining variants, *EXO1* c.1268-1G>T and *FANCC* c.897G>T; p.Arg299Ser, on their gene transcripts has yet to be investigated to support that these variants do indeed affect splicing. This can be performed by different sequencing platforms on cDNA or RNA extracted directly from the PBL of the variant carriers or from the derived LCLs from the carriers' PBL [603]. If that is not possible, another option to assay these variants would be to use a minigene system [540,603,604]. In addition to confirming that the variants above generate alternative transcripts, it is also critical to assess that the splicing event resulting from these variants is not part of the natural occurring process of alternative splicing that produces different, but non-disease causing transcripts [416,605]. It would also be important to investigate whether all these splicing variants along with the other LoF variants I identified, *RAD51D* c.694C>T; p.Arg232Ter (Chapter III) [404] and *NTHL1* c.244C>T; p.Gln82Ter (Chapter V), abrogate the production of the encoded proteins by premature amino acid termination and nonsense-mediated mRNA decay [535]. This can be simply done by assessing protein expression by the conventional Western blotting of cells expressing the variant or by immunohistochemistry of ovarian tumour tissue from the variant carriers, yet this is highly dependent on the specificity of the antibodies. Together, the proposed analyses could provide some insight into the implication of these variants as OC risk candidates [299,416].

In addition to diving deeper into the role of the splicing variants and their potential biological impact, it would also be of interest to study how some of the other variants identified during this work impact cellular function. For instance, in Chapter IV, BRIP1 knock-out cells complemented the BRIP1 c.2990_2993delCAAA; p.Thr997ArgfsTer61 transcript and were shown to express BRIP1 protein, suggesting that the potential associated risk of this variant is not due to the disruption of the production of the

encoded protein as might have been expected. Similarly, it is not known how cell lines expressing RAD51C c.414G>C; p.Leu138Phe, RAD51D c.137C>G; p.Ser46Cys (Chapter III) and BRIP1 c.797C>T; p.Thr266Met and c.2087C>T; p.Pro696Leu (Chapter IV) confer sensitivity to cisplatin. It is critical to investigate the underlying mechanism of how such cellular sensitivity is conferred to support the evidence that these variants increase risk for OC [299]. Moreover, it is not known how both missense variants, NEIL1 c.248G>T; p.Gly83Asp and ERCC5 c.2556A>G; p.Ile852Met, as well as NTHL1 c.244C>T; p.Gln82Ter, EXO1 c.1268-1G>T and FANCC c.897G>T; p.Arg299Ser (Chapter V) impact the encoded proteins in the context of OC. One way to address this is by using the current knowledge of the biological function of EXO1, FANCC and ERCC5 in the HR and FA pathways to assess whether cell lines complemented with these variants confer sensitivity to the OC standard-of-care chemotherapy such as cisplatin or the targeted therapy PARP inhibitors [204,606] as seen in Chapters III [404] and IV. For instance, the biological impact of variants in the DNA glycosylase genes, NEIL1 and NTHL1, that are involved in the base excision repair pathway can be investigated by measuring the accumulation of DNA damage or replication stress in cells expressing the variant compared to the wild-type after being subjected to base-damaging inducing agents [503,607]. All suggested investigations can be performed in multiplex fashion to minimize the time and effort required to perform the assays [193–203]. Currently, considerable effort by multiple research groups is being conducted to screen for all possible missense variants in known and associated OC risk genes (unpublished data presented at Mutational Scanning Symposium 2022 and Cold Spring Harbor Laboratory Symposium 2022). It is important to emphasize the fact that all suggested functional assays will address the biological impact of these specific variants on protein production and thus implicated in risk for OC, and not the cause of OC [299].

In the original research studies, I showed the merit of genetically characterizing candidate variants in known OC risk genes (Chapters II to IV) and identifying recurring candidate variants in new genes that had not previously been implicated in OC risk (Chapter V) in the FC population of Quebec. In order to investigate the spectrum and prevalence of all potentially deleterious variants in these genes in the FC population, WES or WGS can be performed on all FC OC families and sporadic cases and

compared to population-matched controls. The associated risk of all LoF and potentially deleterious missense variants in each gene can then assessed, with a specific focus on the most frequently occurring variants in the FC population. The fact that the CARTaGENE biobank of cancer-free FC controls (cartagene.qc.ca) has over 600 associated variables, including family history of cancer as well as other known epidemiological risk factors [409], makes this a useful resource for the assessment of associated risk for each of these genes. Performing WGS would enable haplotype analyses to determine whether carriers of more than one recurring variants are likely to share a common ancestor and to estimate the age of these shared haplotypes [182].

Variants that were identified throughout WES and bioinformatics analyses in my original research studies (Chapters II to V) merit further investigation as described in the original research studies and in this Chapter. It would be particularly relevant to dive deeper into the study of more of the variants in the various DNA repair genes that I identified (Chapter V) as we selected only the top-ranking variants based on the current knowledge of DNA repair pathways in the context of hereditary cancer syndromes. Taken altogether, these proposed analyses could collectively support the evidence for my candidate variants and/or genes identified in my original research chapters for risk for OC [299].

CHAPTER VII: Conclusion

Applying WES and bioinformatic analyses for my proposed strategies to investigate highly selected OC families and/or cases from a defined population exhibiting founder effect and genetic drift, the FC population of Quebec, I focused on genes involved in DNA repair pathways to identify: (1) likely PVs in the known OC risk genes, *BRCA1*, *RAD51C*, *RAD51D* and *BRIP1*; and (2) potentially deleterious candidate variants in new genes that previously have never been implicated in OC predisposition, *ERCC5*, *EXO1*, *FANCC*, *NEIL1* or *NTHL1*, that may also be relevant to non-FC populations. My customized filtering and prioritizing criteria allowed me to focus on the type of the variant in the context of OC predisposition. Such criteria along with my selected in silico tools allowed me to identify variants that were demonstrated to have a potential impact on the biological function at the level of the RNA transcript or protein by using in vitro or in cellulo assays, supporting their pathogenicity in the context of hereditary OC. Altogether, my approach and genetic analyses of women with OC, who tested negative for *BRCA1* or *BRCA2* PVs, support my hypothesis that they harbour LPVs in other known or new candidate OC risk genes involved in DNA repair pathways.

APPENDICES

Appendix I: Under-investigated founder populations: Insights on hereditary ovarian cancer in the population of the Arabian Peninsula

As I am from one of the under-investigated founder populations, the population of the Arabian Peninsula, specifically the population of Saudi Arabia, I decided to provide some insights on hereditary OC in the Saudi population.

The Arabian Peninsula has been occupied by humans for over 85,000 years [608], and currently comprises the six Gulf Cooperation Council countries: Saudi Arabia, Kuwait, Bahrain, Qatar, Oman, and the United Arab Emirates as well as Yemen and southern regions of Jordan and Iraq [609]. Tribalism has been a deeply rooted tradition in the Arabian Peninsula which is structured as multiple groups, each representing a tribe, who randomly settled in the Arabian Peninsula [609,610]. These tribes have been sharing the common practice of intra-tribal consanguineous marriages [609]. Saudi Arabia occupies the majority of the Arabian Peninsula and tribal individuals account for at least 70% of the Saudi population [609,610] whose settlement in the Arabian peninsula is believed to date back at least 1,000 years [611–613]. The high rate of consanguineous marriages of up to 70% and the high fertility rate have contributed to the unique regional genetic architecture of the Saudi population [609,614,615]. Such consanguineous marriages have been linked to the strong clustering of at least 28 large tribes in Saudi Arabia, each exhibiting a unique genetic architecture, and these clusters correlate with their geographical proximities in Saudi Arabia [610]. It is not surprising then to observe an increased incidence of some autosomal recessive disorders [609,614,616–619]. It has been estimated that a rare variant with a minor allele frequency of 0.001 will be observed as homozygous once per 1,000,000 individuals in the general population, but once per 41,494 individuals in the Saudi population [614]. On the other hand, the incidence of the majority of autosomal dominant syndromes, including hereditary cancer syndromes, is not affected by the consanguinity rate [614,620], as expected.

OC is the seventh most prevalent cancer among women in Saudi Arabia and it accounts for 2.9% of all cancers, which is comparable to those in Western European countries where the proportion is 3.4%, although there are regional differences in the

disease incidence [3,621,622]. OC is also the leading cause of death among gynecological cancers in Saudi Arabia as it is in most Western European countries, and the lifetime probability of dying from OC is estimated to be 1 in 100 Saudi women [3,621,622]. On the other hand, there is a significant difference in the median age at diagnosis of OC among Saudi women (55 years) compared to the Western European populations (62-65 years) [622,623], and a higher incidence of the mucinous subtype (19%) reported in Saudi women with epithelial OC compared to 3% in women from Western European countries [20,622,623]. Hereditary OC is estimated to be approximately 5-15% of all sporadic OC cases (that is, those cases not selected on the basis of a family history of cancer) among the Saudi population [621,624]. Although research on hereditary OC in the Saudi population is in its infancy, there are a few promising recent studies [621,624–627]. It is not surprising that the most studied genes in the context of hereditary OC in the Saudi population are *BRCA1* and *BRCA2* [626]. Currently, there are over 20 different PVs in *BRCA1* and *BRCA2* that have been reported in OC families and cases from the Saudi population using targeted genotyping of specific PVs, or targeted sequencing of whole coding and flanking regions of *BRCA1* and *BRCA2* exons [621,624,625]. Approximately two-thirds of these variants in *BRCA1* and *BRCA2* have been observed in other populations, including Western European countries. Interestingly, less than ten of these variants, all in *BRCA1*, were found in at least two OC cases [621], four of which have never been reported in ClinVar (last accessed on July 4, 2022) or have been identified in gnomAD v2.1.1 and v3.1.2. Haplotype analyses of these four variants revealed that carriers of two out of four variants in *BRCA1* (c.1140dupG; p.Lys381GlufsTer3 and c.4136_4137delCTinsAA; p.Ser1379Ter) shared the same haplotype with a length of 1.04 and 1.46 megabase, respectively, suggesting that these each of these frequently occurring variants was inherited from a common ancestor [621,628]. The carrier frequency of these two variants account for 42% (21/50) of all *BRCA1* or *BRCA2* PVs in sporadic OC cases and 5.2% (21/407) of all sporadic OC cases regardless of *BRCA1* and *BRCA2* status. To date, many fundamental questions still need to be addressed in terms of the contribution of PVs in *BRCA1* and *BRCA2* in the context of HBOC versus sporadic cases and an in-depth analysis of the associated phenotypes with OC carriers of PVs in both *BRCA1*

and *BRCA2* has yet to be performed. This would be beneficial in medical genetic settings, specifically for referral of patients that may require further assessment and management. The contribution of PVs in other known OC risk genes such *MLH1*, *MSH2*, *MSH6*, *PMS2*, *BRIP1*, *RAD51C*, and *RAD51D* is also not known. Only one study expanded the genetic analysis to 55 genes in cases with different cancer syndromes, including 118 sporadic OC cases [624]. In this analysis, one PV in *RAD51C*, c.52_53del; p.Pro18AlafsTer18, was identified in an OC case [624], but this variant was not identified in gnomAD v2.1.1 or v3.1.2, but was reported once in ClinVar (last accessed July 4, 2022). Interestingly, the same study identified two OC cases (1.7%) harbouring two PVs in *TP53*, c.524G>A; Arg175His and c.563C>A; p.Ser188Tyr [624], a gene associated with Li-Fraumeni syndrome (MIM:151623). This suggests a relatively high carrier frequency of PVs in *TP53* in the Saudi population, given the high frequency of BC carriers of PVs in *TP53*, a known BC risk gene, of approximately 1% (7/698) [624] compared to approximately 0.02% in BC carriers from Western Europe [629].

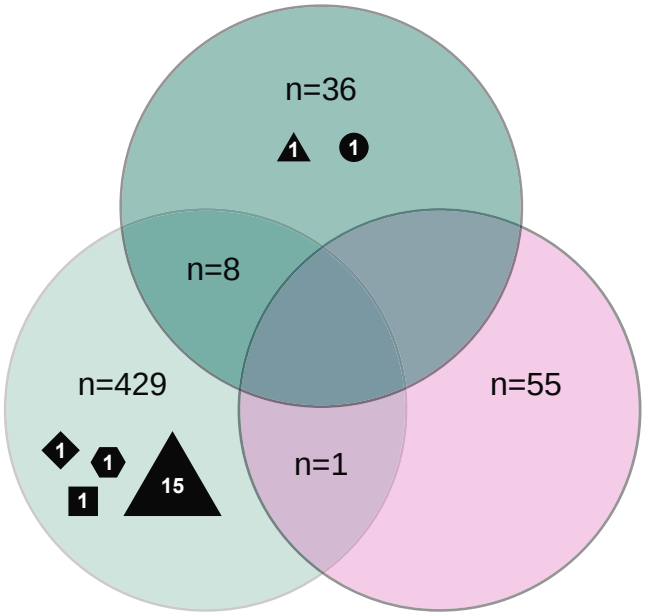
Although there is huge gap in our knowledge of the genetic landscape of various hereditary cancer syndromes including OC in the Saudi population, investigating these cancers from such populations with unique genetic architectures could provide some insight into the characteristics of rare PVs in known CPGs and the possibility of identifying new candidate cancer risk genes. This could lead to earlier diagnosis of inherited cancers and better clinical management in the Saudi population.

Appendix II: CHAPTER II supplementary materials

See submitted file Appendix_II_CHAPTER_II_Suppl_Tables.xlsx

Appendix III: CHAPTER III supplementary materials

See submitted file Appendix_III_CHAPTER_III_Suppl_Tables.xlsx



Study groups:

- OC families (n=44)
- HBOC families (n=56)
- Sporadic OC cases (n=438)

Variant carriers:

- ● *RAD51C* c.414G>C
- ⬣ *RAD51C* c.705G>T
- ■ *RAD51D* c.137C>G
- ◆ *RAD51D* c.694C>T
- ▲ *RAD51D* c.620C>T

Figure S3.1. Venn diagram depicting relationship between carriers of candidate *RAD51C* or *RAD51D* variants in study groups investigated in phase II.

Each circle contains the total number (n) of cases investigated in ovarian cancer (OC) families, Hereditary Breast and Ovarian Cancer (HBOC) syndrome or sporadic disease study groups (see **Table S3.1.**). The number of cases appearing in overlapping circles denote identical cases known to have been recruited to these study groups. The number inside each black symbol contains the carriers of each specific variant identified.

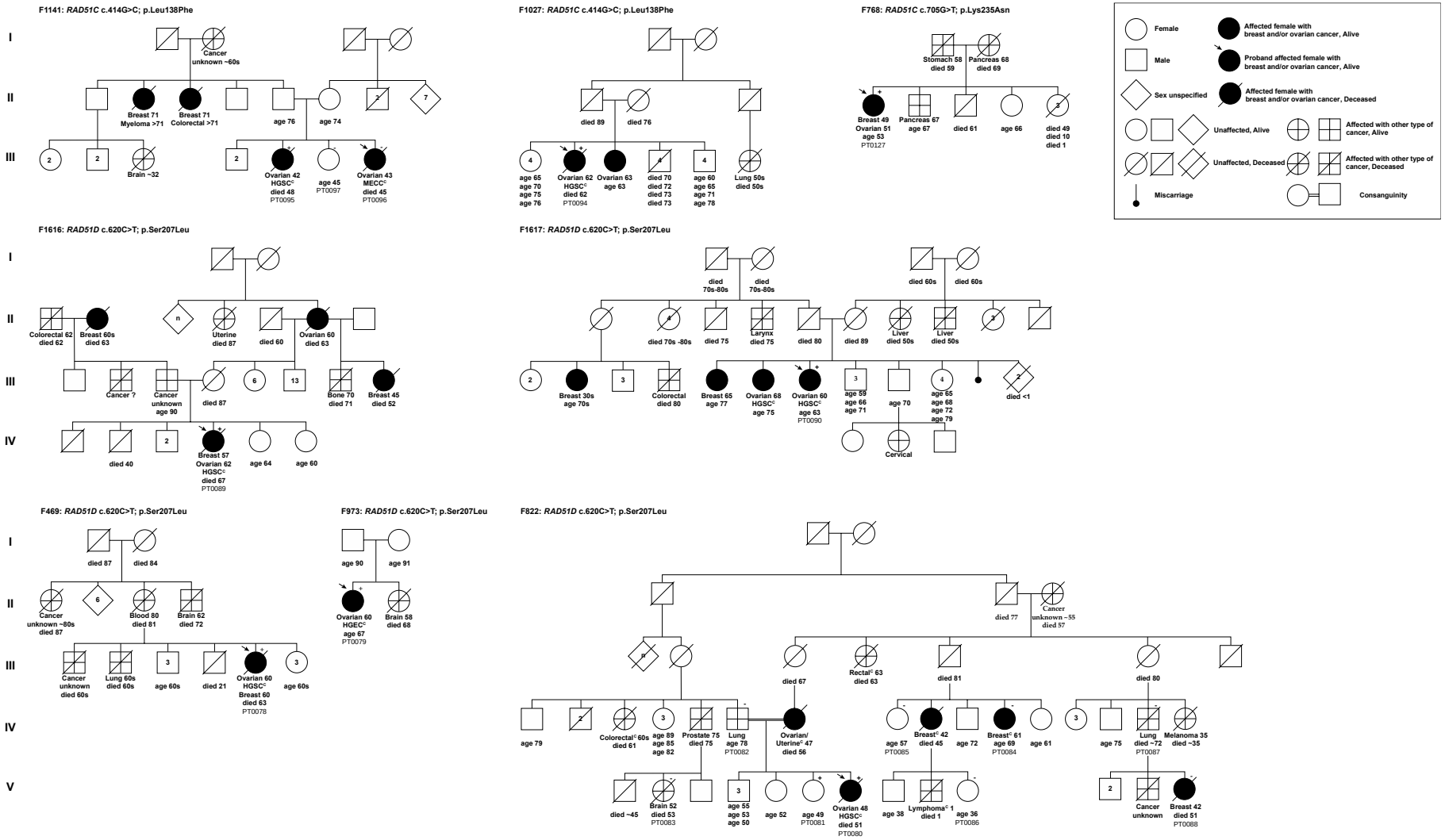


Figure S3.2. Pedigrees of selected index ovarian cancer cases carrying a candidate *RAD51C* or *RAD51D* variant.

Cases from family number F1616, F1617 and F1141 were part of phase I of the study, those from F822, and F1027 were part of phase II, and F469, F768 and F973 were part of phase III. Carrier status of index tested (arrow) and additional available family members are denoted by plus (carrier) or minus sign. All carriers were found in a heterozygous state. Age in years is shown with cancer diagnosis (HGSC: High-grade serous carcinoma of the ovary; MECC: Mixed high-grade endometrioid carcinoma of the ovary with clear cell) or death. Superscript C denotes histological subtypes that were confirmed by pathology reports or death certificates. Both pedigrees and case number tested were anonymized to protect the identities of the participants.

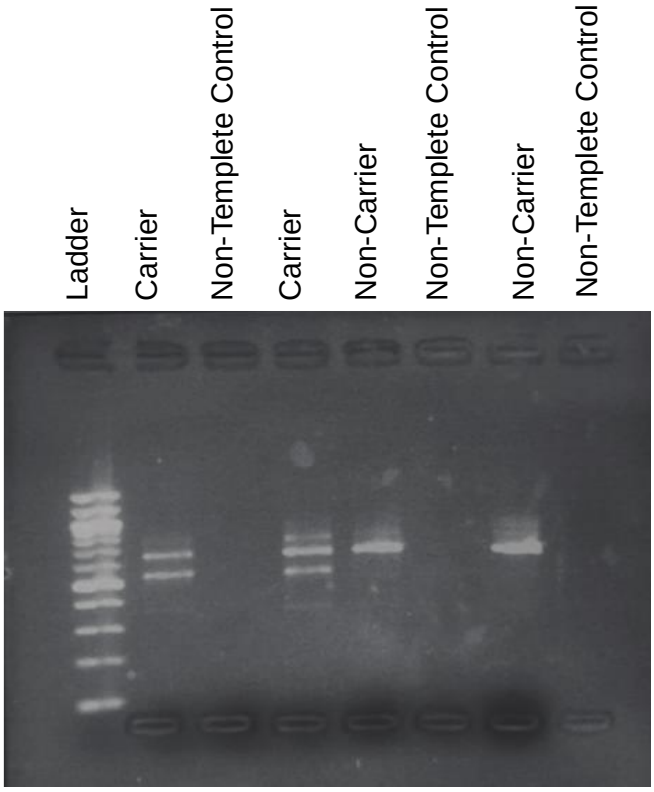


Figure S3.3. Uncropped gel electrophoresis of carrier- and non-carrier-derived lymphoblastoid cell lines of *RAD51C* c.705G>T.

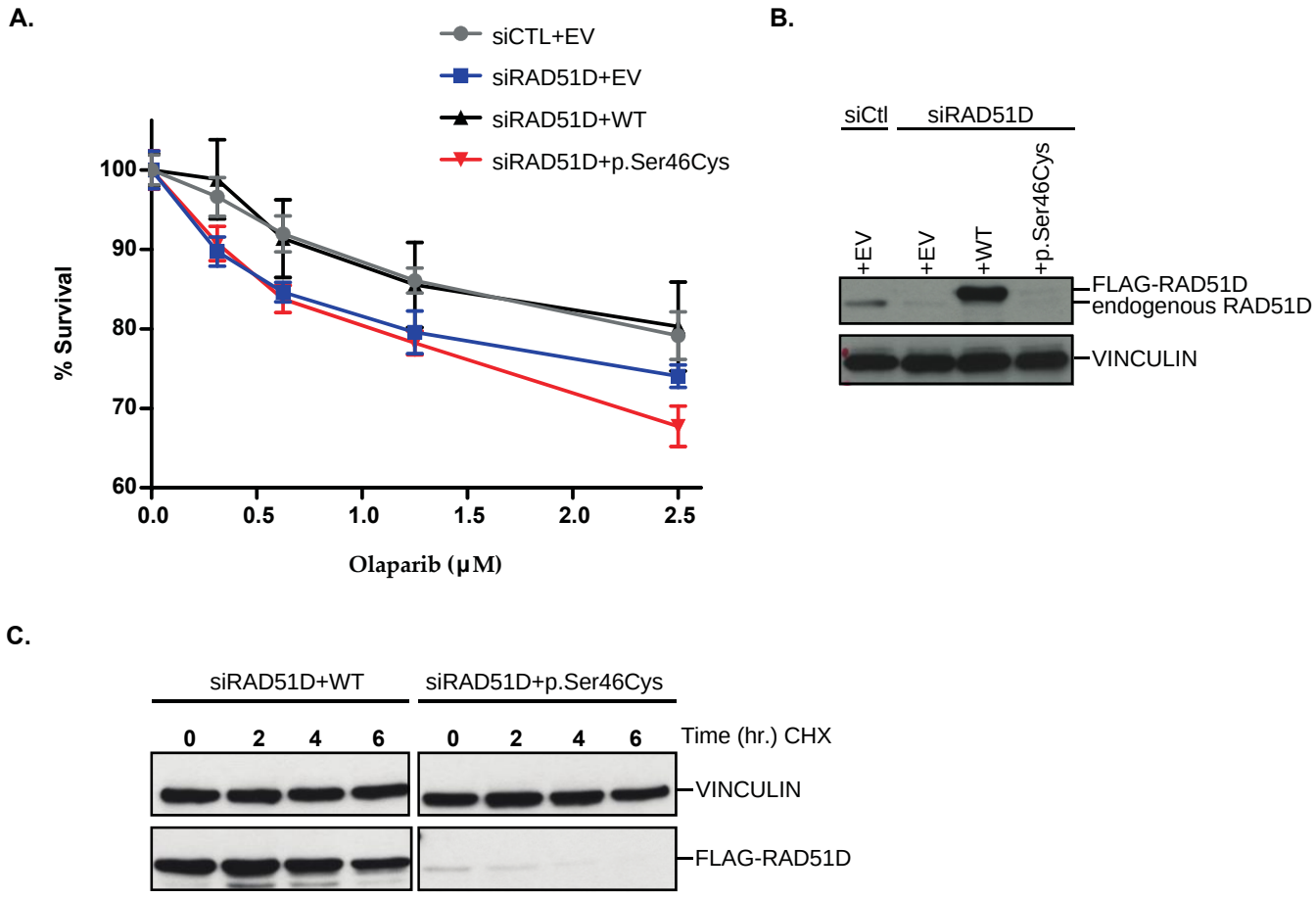


Figure S3.4. In cellulo functional characterization of the RAD51D p.Ser46Cys variant in the Hela cells

RAD51D-knockdown Hela cells, previously transfected with the empty vector (EV) or the indicated siRNA-resistant RAD51D constructs, including siRNA non-targeting controls (siCTL), were seeded in 96-well plates and exposed to increasing concentrations of olaparib. Cell viabilities were obtained from the 96-well plates post-treatment by quantification of surviving Hoechst-stained nuclei and represented in percentage of survival relative to the control (DMSO-treated) condition; (A) Viability curves contrasting the abilities of RAD51D wild-type and the p.Ser46Cys variant to rescue olaparib resistance to RAD51D-knockdown cells. Data is presented as the mean (± standard error mean [SEM]) from at least three independent experiments, each performed in triplicate; (B) RAD51D levels of after knockdown and re-expression in HeLa cells, with vinculin as loading control; (C) RAD51D-knockdown cells were transfected with FLAG-

RAD51D wild-type or FLAG-RAD51D p.Ser46Cys. RAD51D protein levels were assessed at 2, 4, and 6 hours following addition of Cycloheximide (CHX) block. Vinculin was used as loading control.

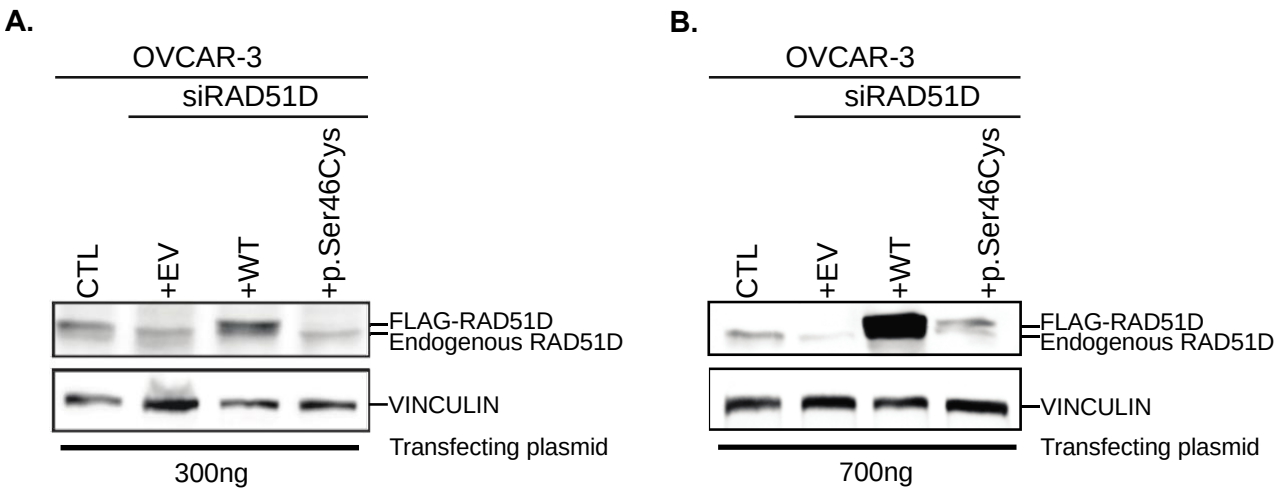


Figure S3.5. The RAD51D p.Ser46Cys variant impairs protein stability in the ovarian cancer cell line OVCAR-3.

Western blots of OVCAR-3 cells transfected with siRNA non-targeting control (siCTL) or targeting RAD51D (siRAD51D) and then complemented with FLAG-RAD51D constructs or empty vector (EV). (A) Cells transfected with 300ng of indicated plasmids; and (B) Cells transfected with 700ng of indicated plasmids. Vinculin was used as loading control.

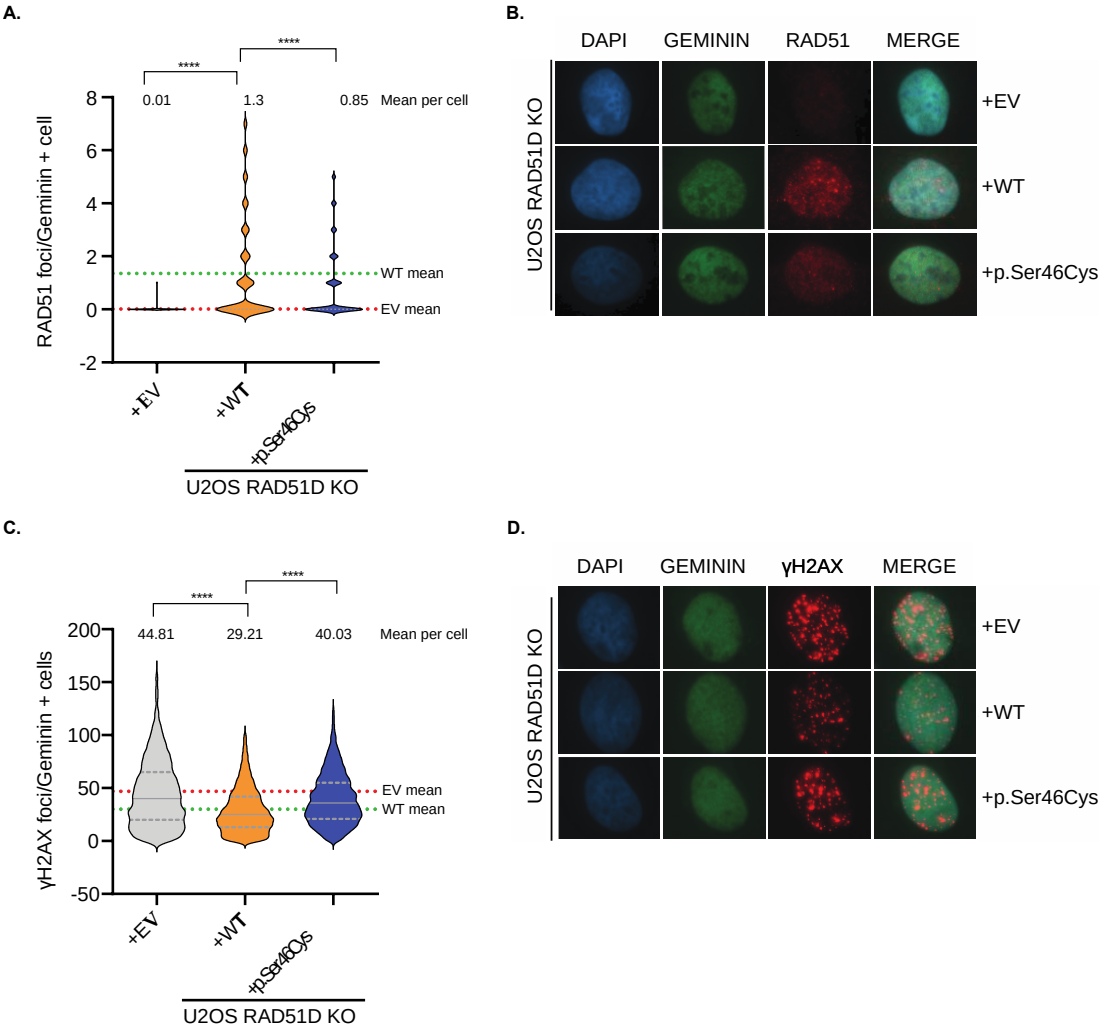


Figure S3.6. The RAD51D p.Ser46Cys variant impacts RAD51 and γH2AX foci formation in U2OS RAD51D knock-out cells.

(A-D) Immunofluorescence of U2OS RAD51D knock-out (KO) cells complemented with the indicated RAD51D constructs of RAD51D. Experiments have been performed in triplicate. Statistical significance was determined by Kruskal-Wallis test with Dunn's multiple comparison post-test (****P<0.0001) (A) Violin plots shows the number of RAD51D foci in Geminin-positive cells 4h after 5 Gray irradiation. (B) RAD51D immunofluorescence representative images. (C) Violin plot shows the number of γH2AX foci in Geminin-positive cells 4h after irradiation with 5Gray. (D) γH2AX immunofluorescence representative images. Dashed grey lines represent quartiles and the median is depicted with gray lines.

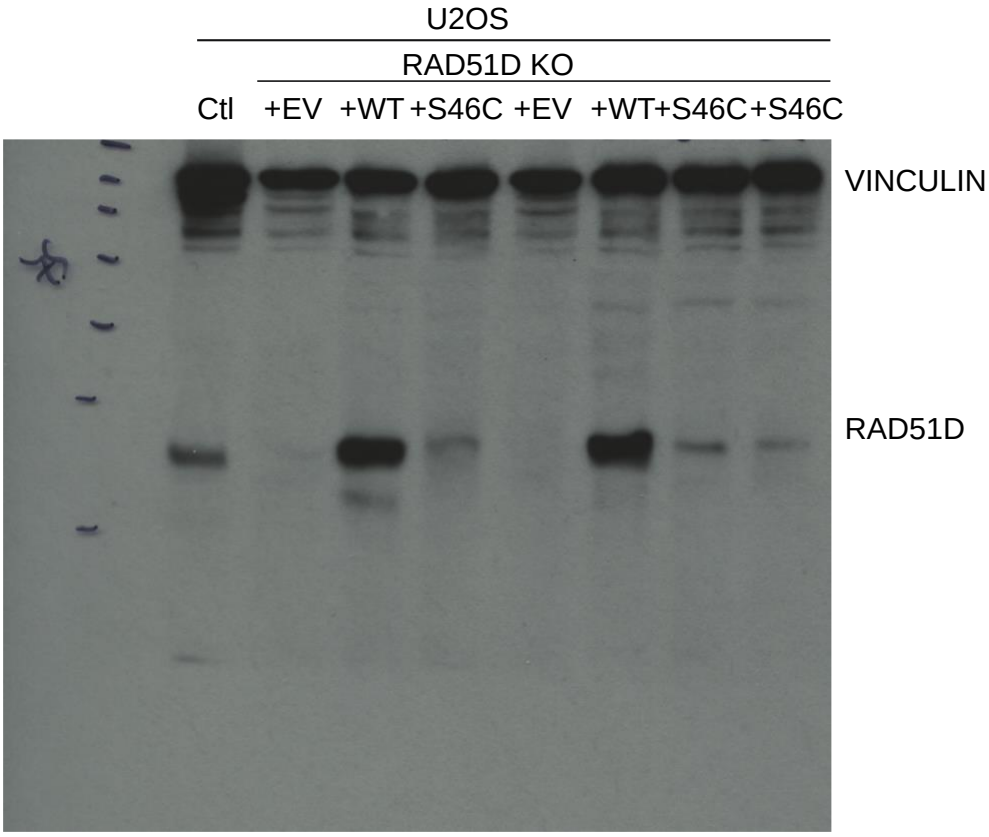


Figure S3.7. Uncropped western blot of U2OS RAD51D knock-out cells stably and complemented with wild-type or RAD51D p.Ser46Cys.

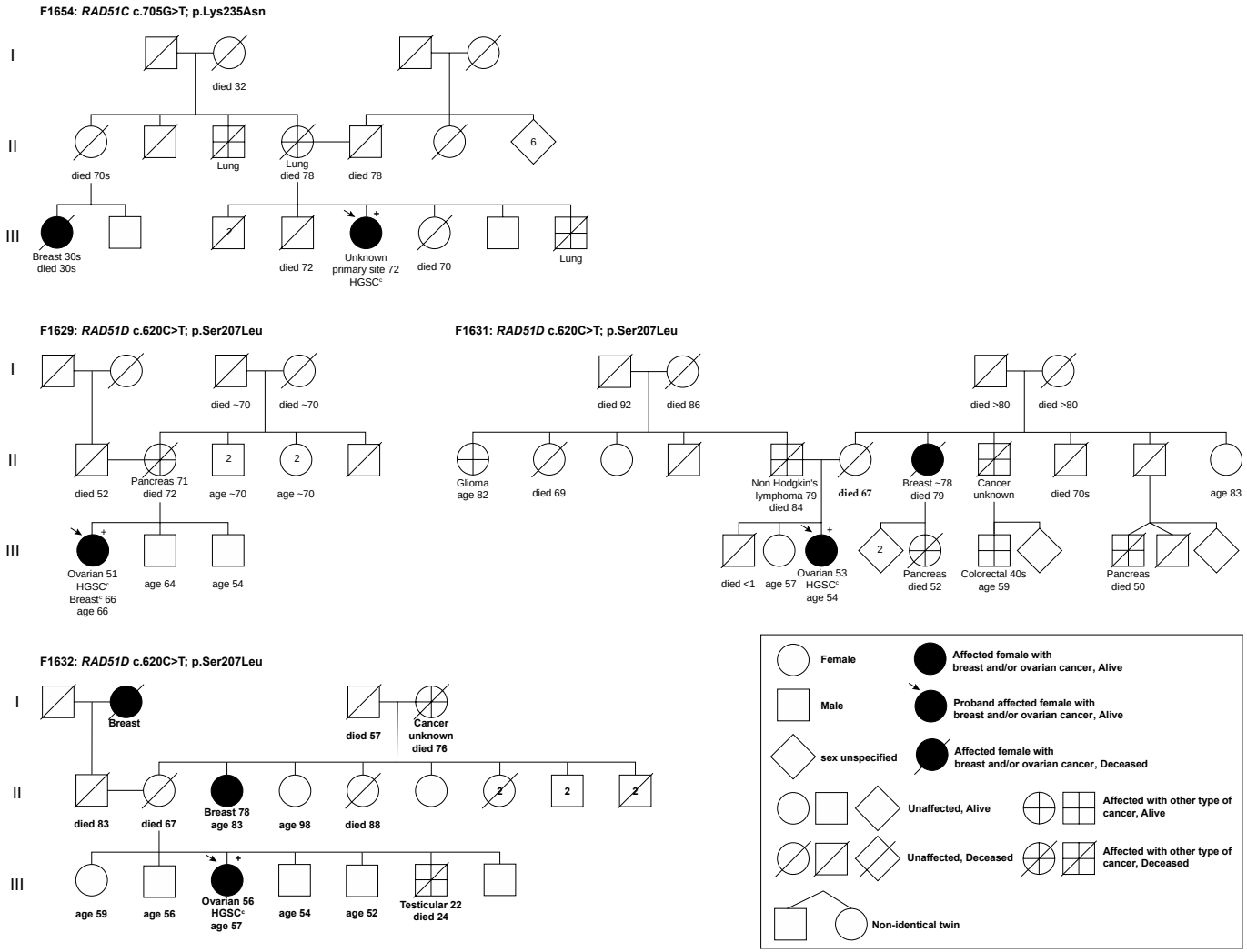


Figure S3.8. Additional pedigrees of ovarian cancer cases carrying a candidate *RAD51C* or *RAD51D* variant.

Carrier status of index case tested (arrow) denoted by a plus (carrier) or minus sign. All carriers were reported to be in heterozygous state. Age in years is shown with cancer diagnosis (HGSC: High-grade serous carcinoma of the ovary) or death.

A review of pathology report of the index tested case in pedigree number F1654 confirmed a diagnosis of high-grade serous carcinoma of unknown origin (likely upper genital tract). Superscript C denotes histopathology was confirmed by pathology reports or death certificates. Pedigrees were anonymized to protect the identities of the participants.

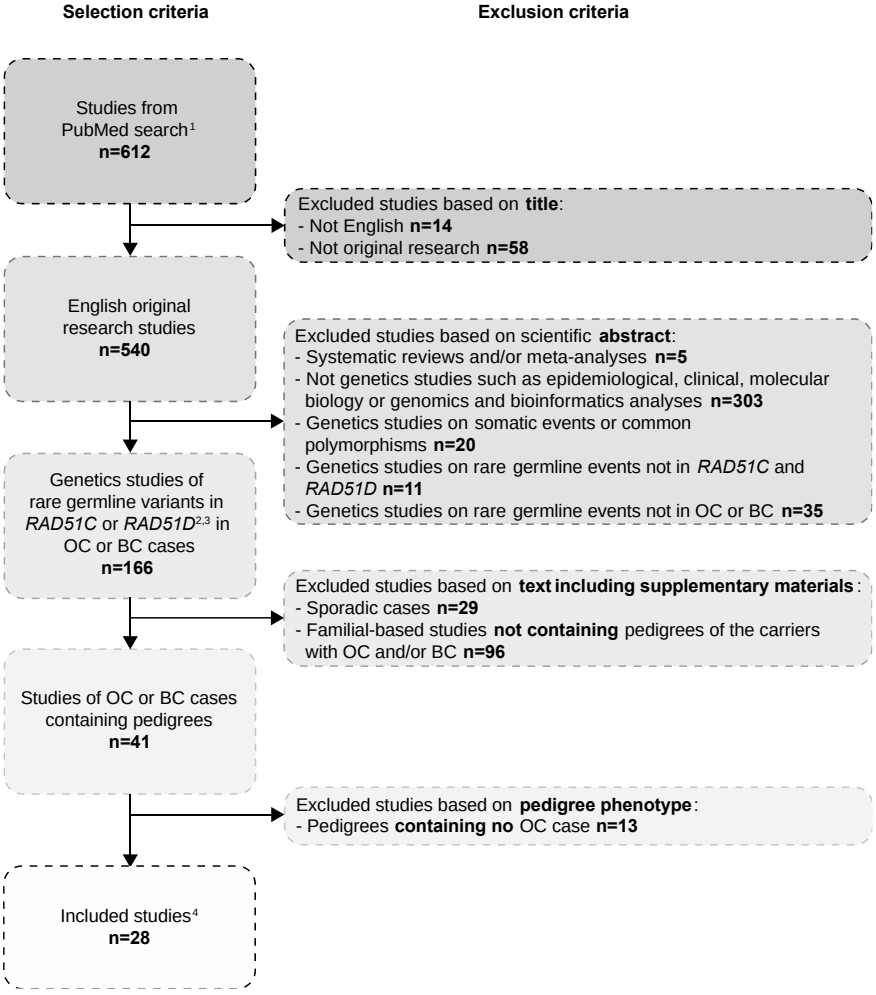


Figure S3.9. Schematic diagram of the criteria used for selecting rare *RAD51C* or *RAD51D* variants implicated in familial ovarian cancer from the published literature.

[1] PubMed database (pubmed.ncbi.nlm.nih.gov) was searched for articles up to October 2021 using the following terms: “RAD51C"[All Fields]; “FANCO"[All Fields]; “RAD51L2"[All Fields]; “RAD51D"[All Fields]; or “RAD51L3"[All Fields].

[2] Germline substitutions and small or large deletions or insertions variants were included.

[3] Germline variants classified as uncertain significance as reported by ClinVar and The American College of Medical Genetics and Genomics (ACMG) were excluded.

[4] Data from these studies appear in **Figure S3.5**.

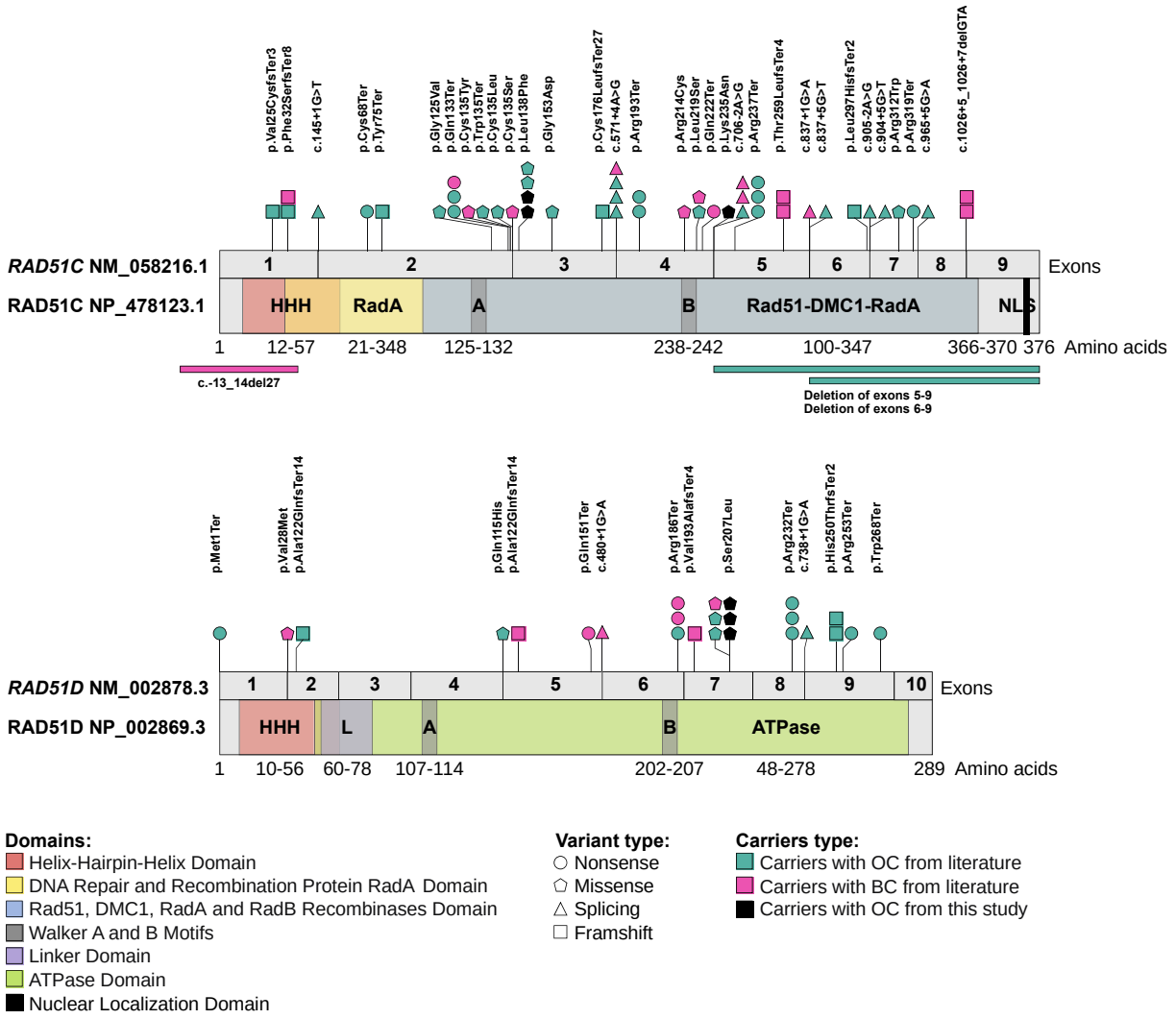


Figure S3.10. Lollipop figure of *RAD51C* and *RAD51D* functional protein domains with reported germline variants.

Lollipop depicting the location of all reported germline variants in published pedigrees of carriers with ovarian (OC) or breast cancer (BC) cases with a family history of OC in known protein domains of *RAD51C* at the mRNA (NM_058216.1) and protein (NP_478123.1) level (upper panel) and *RAD51D* at mRNA (NM_002878.3) and protein (NP_002869.3) level (lower panel) based on the NCBI Reference Sequence (RefSeq) database (ncbi.nlm.nih.gov/refseq/).

Appendix IV: Permission to include a manuscript as Chapter IV from first co-author.

From: Larissa Milano, Department of Molecular Biology, Medical Biochemistry and Pathology, Laval University Cancer Research Center and Genome Stability Laboratory, CHU de Québec Research Center.
Date: 14/07/2022

To whom it may concern,

Permission to include manuscript in thesis

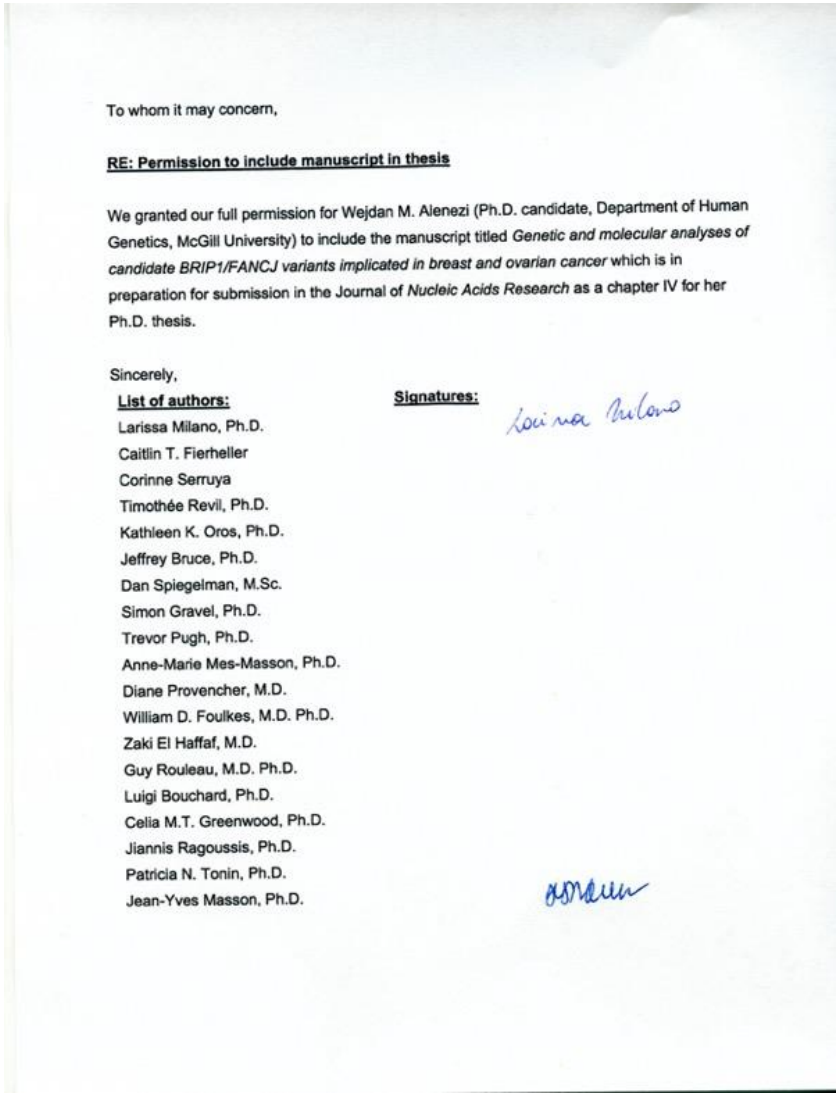
I am writing to confirm that Wejdan M. Alenezi (Ph.D. candidate, Department of Human Genetics, McGill University) and I are co-first authors of the manuscript titled *Genetic and molecular analyses of candidate BRIP1/FANCJ variants implicated in breast and ovarian cancer* which is in preparation for submission in the Journal of *Nucleic Acids Research*.

This original research article includes work to be included as a chapter IV of Wejdan's Ph.D. thesis; thus, she requested for my permission to include this work in her thesis. I have accordingly granted her my full permission to include this work in her thesis.

Sincerely,

Larissa Milano, Ph.D.

Appendix V: Permission to include a manuscript as Chapter IV from co-authors.



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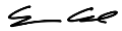
Dan Spiegelman, M.Sc.	
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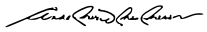
Anne-Marie Mes-Masson, Ph.D.	
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Appendix VI: CHAPTER IV supplementary materials

See submitted file Appendix_VI_CHAPTER_IV_Suppl_Tables.xlsx

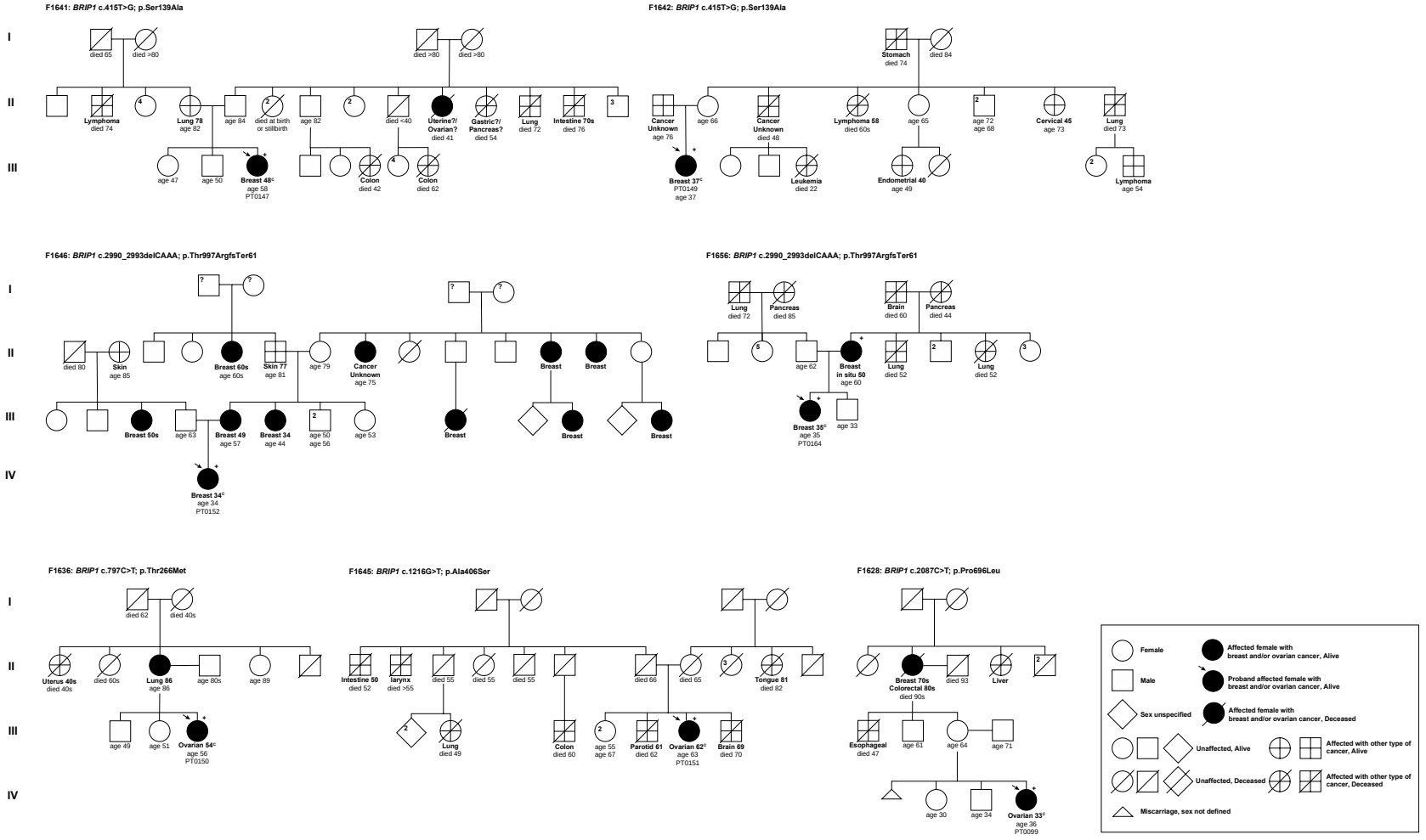

Figure S4.1. Anonymized pedigrees of index ovarian or breast cancer cases carrying a candidate BRIP1 variant reported by the adult hereditary cancer clinics.

Carrier status of index cases (arrow) tested positive are denoted by plus sign. All carriers were found in a heterozygous state. Age in years is shown with cancer diagnosis or death. Unconfirmed cancer status as reported by the index case was denoted by a question mark (?) beside the reported cancer. All breast cancer (BC) cases were invasive unless stated otherwise (see **Table S4.5.**). Superscript C denotes histological subtype that was confirmed by pathology report.

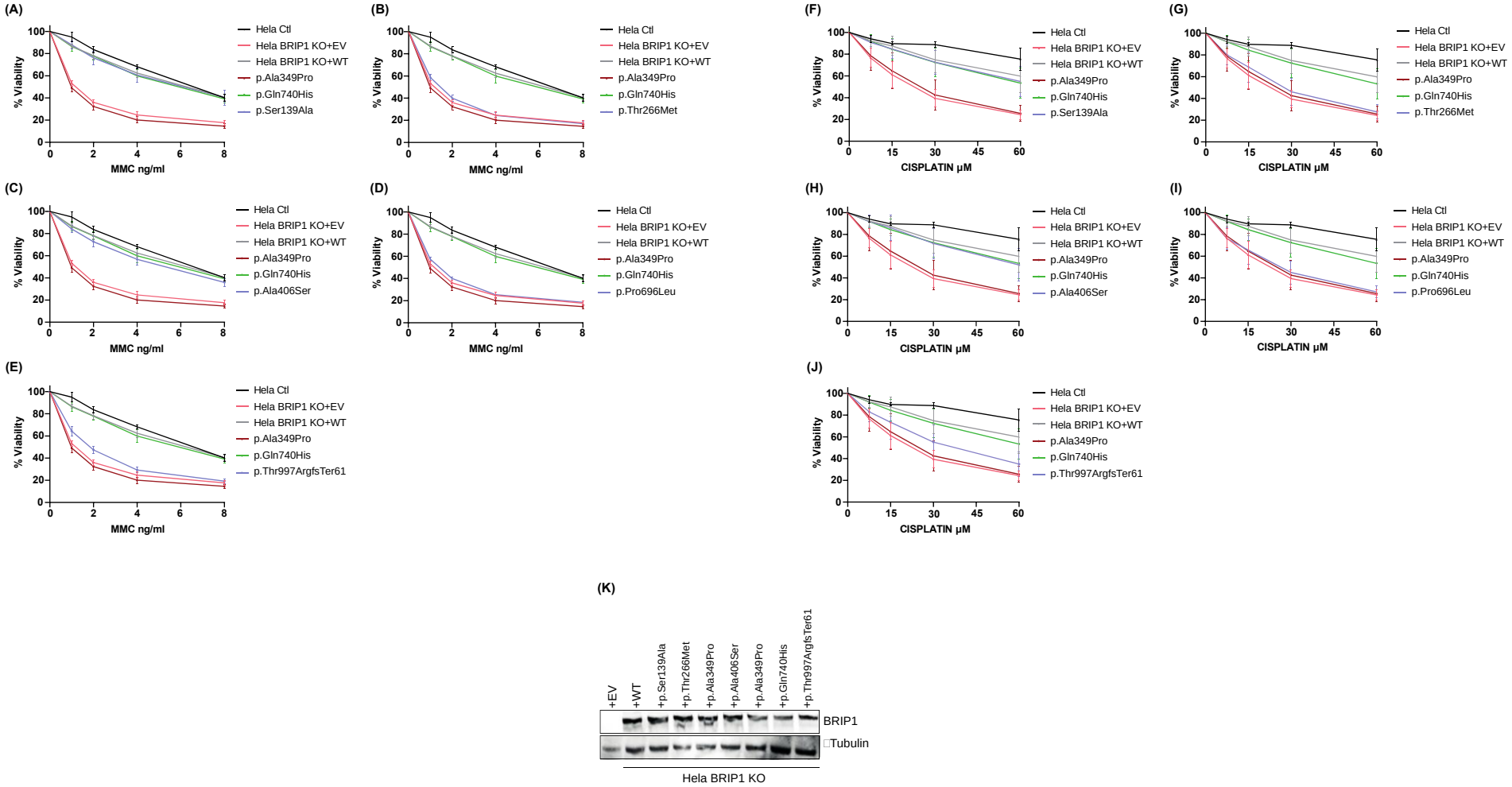

Figure S4.2. Sensitivity curves of BRIP1 variants to DNA inter- or intra-strand crosslinks inducing agents in Hela cells. Survival curves contrasting the abilities of BRIP1 wild-type (WT) and the indicated variants, including the empty vector (EV), to rescue mitomycin C (MMC) (A-E) and cisplatin (F-J) resistance in Hela BRIP1-depleted cells. (K) Western blot representing expression of the indicated variants in Hela BRIP1 depleted cells.

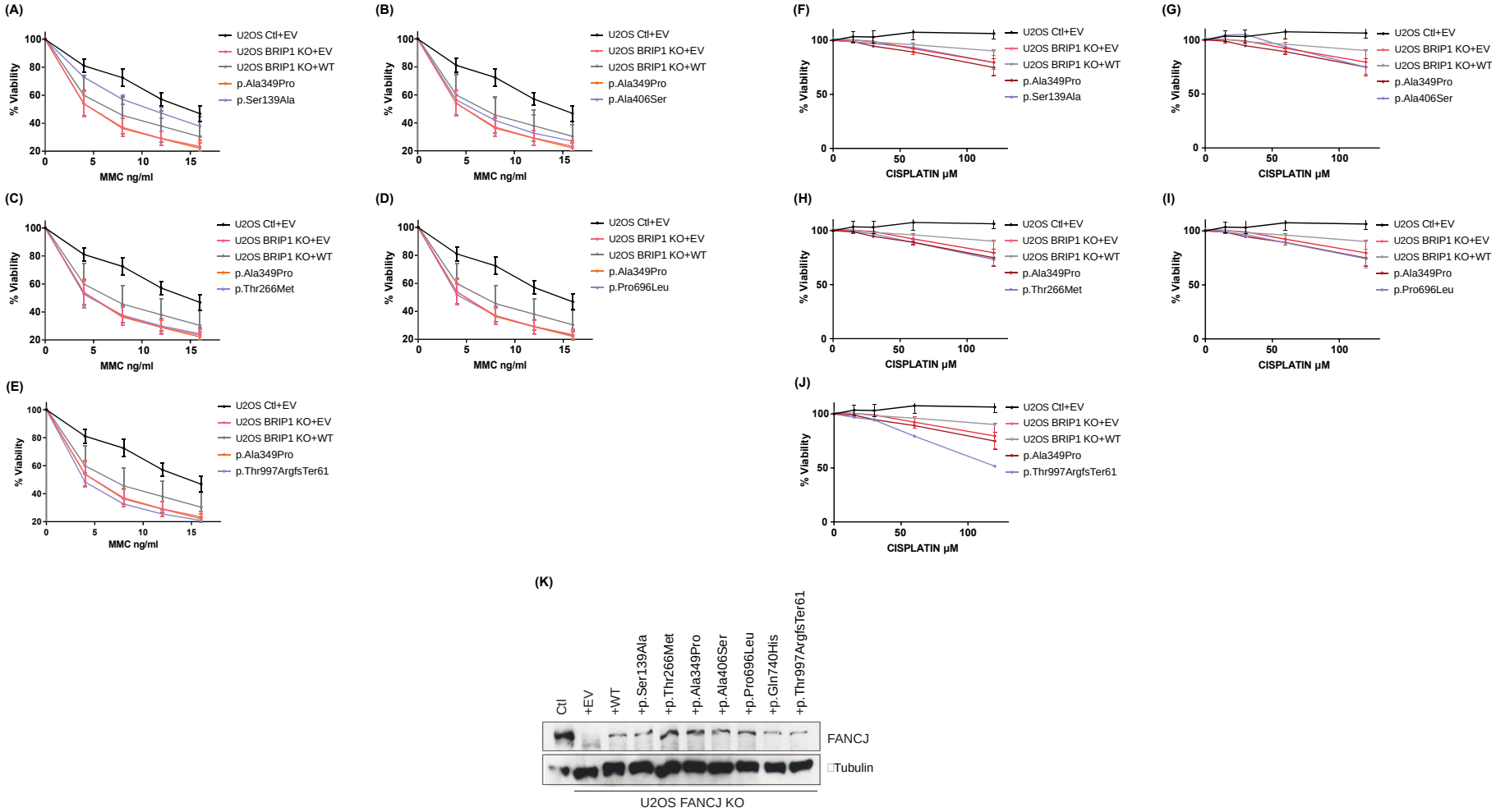

Figure S4.3. Sensitivity curves of BRIP1 variants to DNA inter- or intra-strand crosslinks inducing agents in U2OS cells.

Survival curves contrasting the abilities of BRIP1 wild-type (WT) and the indicated variants, including the empty vector (EV), to rescue mitomycin C (MMC) (A-E) and cisplatin (F-J) resistance in U2OS BRIP1-depleted cells. (K) Western blot representing expression of the indicated variants in U2OS BRIP1 depleted cells.

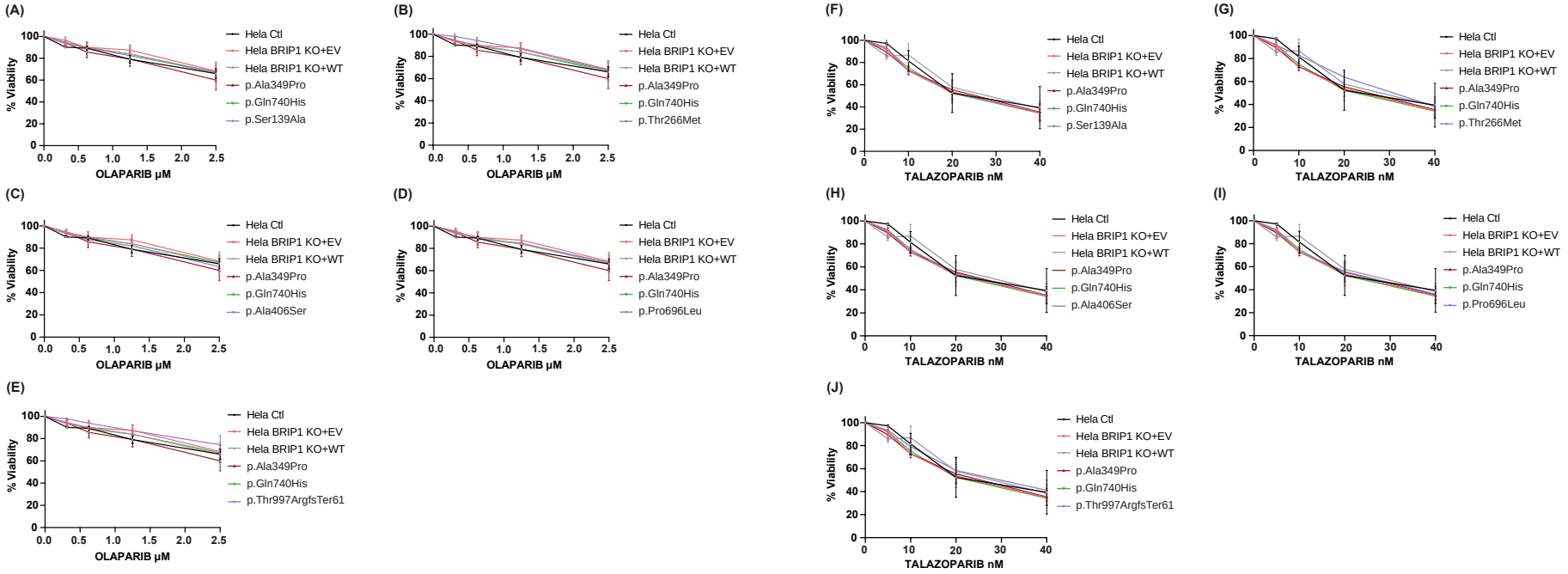

Figure S4.4. Sensitivity curves of BRIP1 variants to poly-ADP-ribose polymerase inhibitors in Hela cells.

Survival curves contrasting the abilities of BRIP1 wild-type (WT) and the indicated variants, including the empty vector (EV), to rescue PARP inhibitors: olaparib (A-E) and talazoparib (F-J) resistance in Hela BRIP1-depleted cells.

Appendix VII: Permission to include a manuscript as Chapter V from co-authors.

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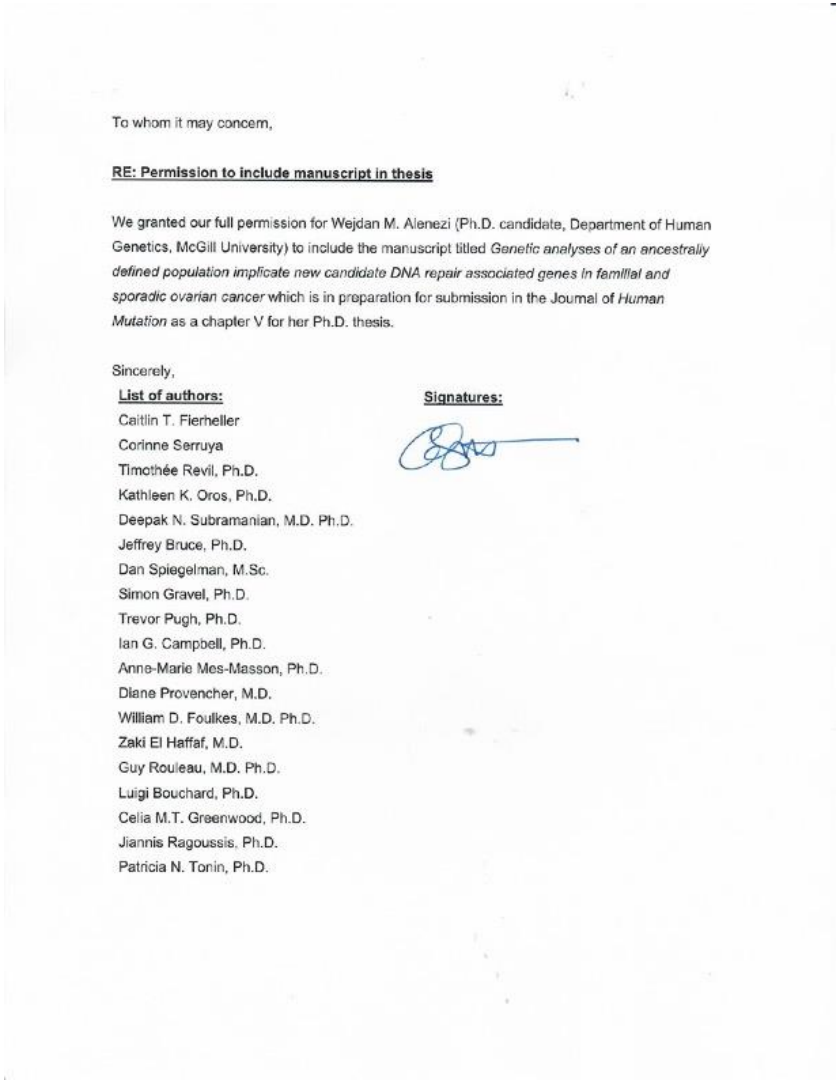

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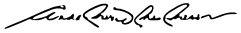
Anne-Marie Mes-Masson, Ph.D.	
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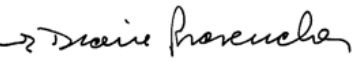
Diane Provencher, M.D.	
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Luigi Bouchard, Ph.D.	
Celia M.T. Greenwood, Ph.D.	
Jiannis Ragoussis, Ph.D.	
Patricia N. Tonin, Ph.D.	

To whom it may concern,

RE: Permission to include manuscript in thesis

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Appendix VIII: CHAPTER V supplementary materials

See submitted file Appendix_VI_CHAPTER_V_Suppl_Tables.xlsx

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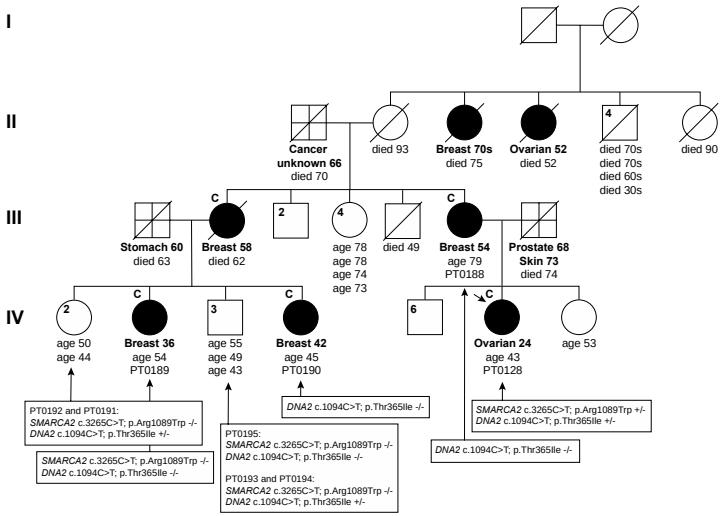

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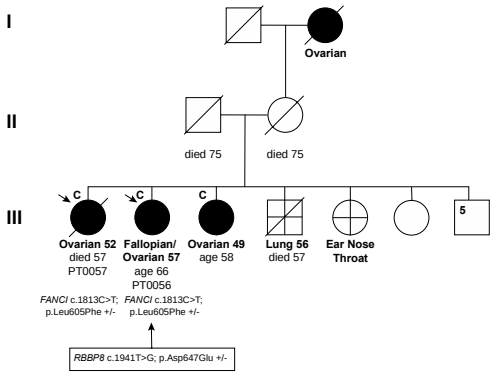

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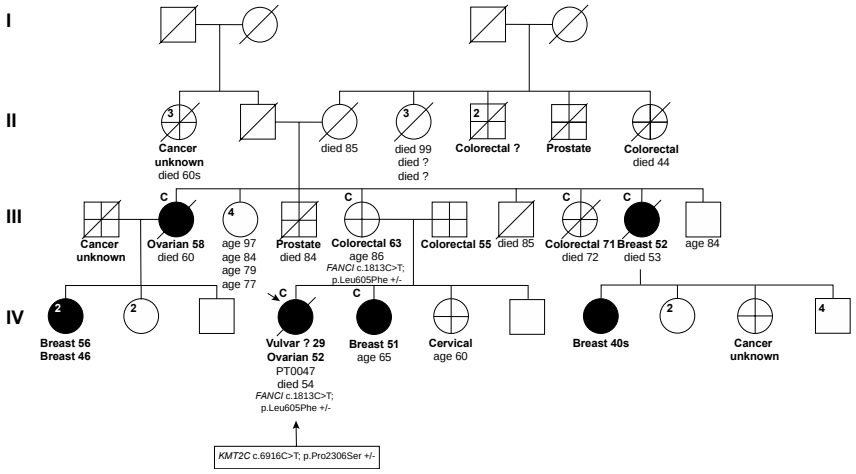

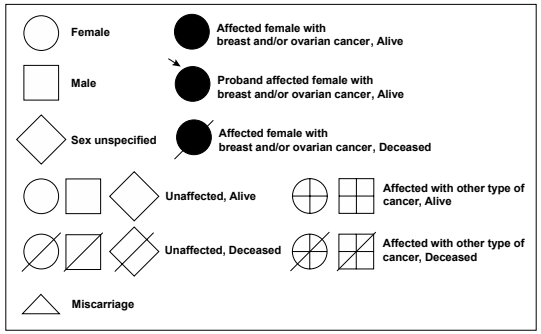

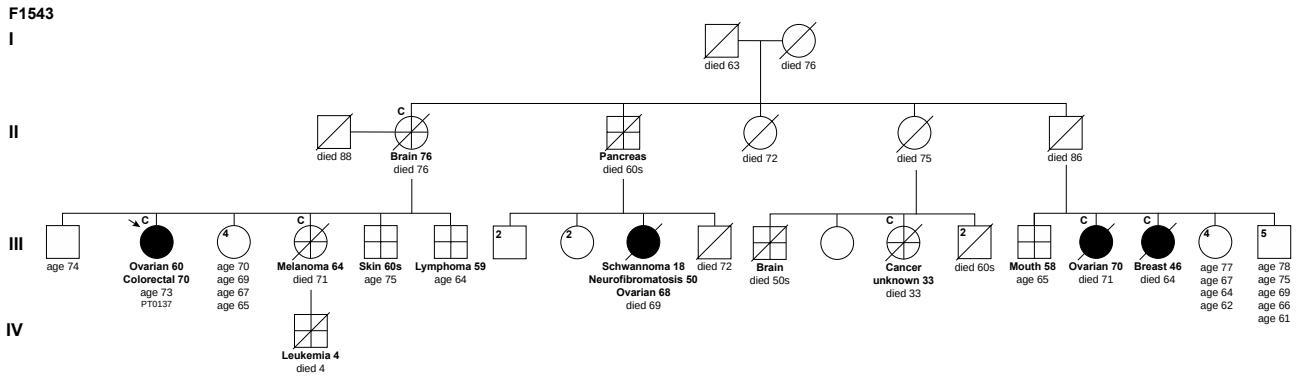
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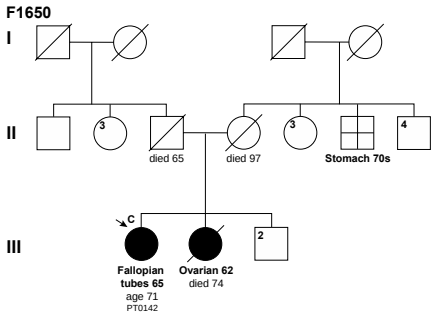
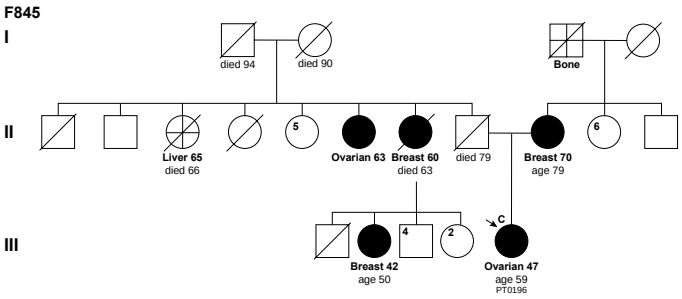
I
II
III
IV
died 63
died 76
died 88
C
Brain 76
died 76
Pancreas
died 60s
died 72
died 75
died 86
age 74
C
Ovarian 60
Colorectal 70
age 73
PT0137
4
age 70
age 69
age 67
age 65
C
Melanoma 64
died 71
Skin 60s
age 75
Lymphoma 59
age 64
Leukemia 4
died 4
2
2
Schwannoma 18
Neurofibromatosis 50
Ovarian 68
died 69
died 72
Brain
died 50s
C
Cancer
unknown 33
died 33
2
died 60s
Mouth 58
age 65
C
Ovarian 70
died 71
C
Breast 46
died 64
4
age 77
age 67
age 64
age 62
5
age 78
age 75
age 69
age 66
age 61


F1650
I
II
III
3
died 65
died 97
3
4
Stomach 70s
C
Fallopian
tubes 65
age 71
PT0142
Ovarian 62
died 74
2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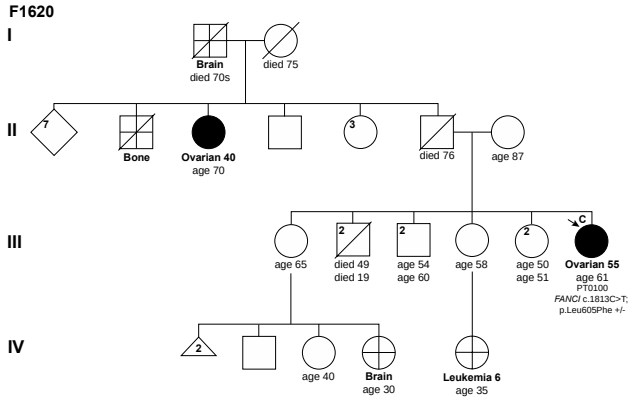
I
II
III
died 94
died 90
Bone
Liver 65
died 66
5
Ovarian 63
Breast 60
died 63
died 79
Breast 70
age 79
6
4
2
Breast 42
age 50
C
Ovarian 47
age 59
PT0196


F1620
I
II
III
IV
Brain
died 70s
died 75
7
Bone
Ovarian 40
age 70
3
died 76
age 87
age 65
2
died 49
died 19
2
age 54
age 60
age 58
2
age 50
age 51
C
Ovarian 55
age 61
PT0100
FANCI c.1813C>T;
p.Leu605Phe +/-
2
age 40
Brain
age 30
Leukemia 6
age 35

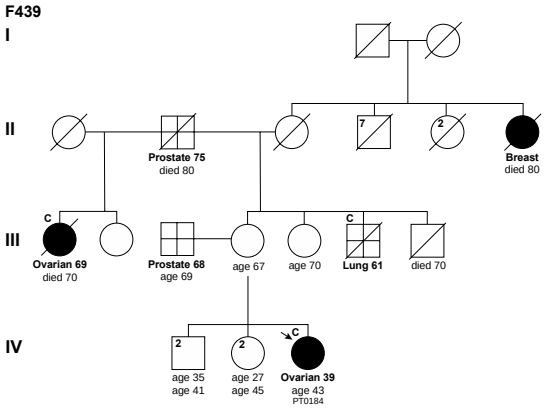

F439
I
II
III
IV
Prostate 75
died 80
7
2
Breast
died 80
C
Ovarian 69
died 70
Prostate 68
age 69
age 67
age 70
C
Lung 61
died 70
2
age 35
age 41
2
age 27
age 45
C
Ovarian 39
age 43
PT0184

Figure S5.1. Pedigrees of index ovarian cancer cases each harbouring candidate variants in DNA repair genes in phase I of the study.

Index cases (arrow) subjected to whole exome sequencing analyses (WES), and carrier status of tested index cases and available family members are denoted by plus (carrier) or minus (not a carrier) signs. All carriers were found in a heterozygous state. Age in years is shown at cancer diagnosis and death where applicable. Superscript C denotes histological subtypes that had been confirmed by pathology reports or death certificates at source. Pedigrees of families: F1490, F1620 and F1528 positive for a likely pathogenic variant *FANCI* c.1813C>T; p.Leu605Phe have been reported previously [384].

Appendix IX: Ethics Board Approval

Centre universitaire de santé McGill — McGill University Health Centre

Annual renewal submission form - Harmonized

Protocol title: **Discovering new high-risk ovarian cancer predisposing genes through targeted genetic analyses of a Canadian founder population**

Project number(s): **MP-37-2019-4783**
Nagano identifier: **CPG-Ovarian Cancer**
Principal investigator: **Patricia Tonin**
Project's REB approbation date: **2018-07-25**

Form: **F9H-96600**
First submit date: **2022-06-09**
Last submit date: **2022-06-09**
Form status: **Form approved**

Administration - REB

1. **MUHC REB Panel & Co-chair(s):**
   Cells, tissues, genetics & qualitative research (CTGQ)
   Co-chair: Marie Hirtle
   reb.ctgq@muhc.mcgill.ca

2. **REB Decision:**
   Approved - REB delegated review

3. **Comments on the decision:**

   Re-approval valid for the following sites:
   - MUHC
   - CIUSSS du Centre Ouest-de-l'Île-de Montréal

4. **Renewal Period Granted:**
   From 2022-07-26 to 2023-07-25

5. **Date of the REB final decision & signature**

2022-07-25

Signature

Sheldon Levy
MUHC REB Coordinator
for MUHC REB Co-chair mentioned above
2022-07-25 11:18

General information

1. **Indicate the name of the Principal Investigator in our institution (MUHC)**

Tonin, Patricia

From which department is the principal investigator?

Medicine

Required information for renewal

1. **Date when the research project is expected to end at your institution:**

Date unknown

Please indicate (approximately) in what year you expect the project to end.

31-03-2025

2. **Indicate the current status of the research project at your institution:**

Project is in progress and recruitment is ongoing

3. **Briefly describe in a few lines, the current status of the project:**

Data analyses is ongoing regarding DNA sequencing (whole exam sequencing analyses), and association with clinical and familial cancer metrics.

4. **Please indicate the type of "participants" implicated in your research project**

Human samples

Did you obtain all the data / samples you needed for the realization of your project as described in the protocol?

No

Please specify

Based on our genetic analyses of individuals there may be additional cases where clinical information and bio samples (cancer specimens) may be investigated for our genetic variants of interest (as this is a discovery project).

5. **In terms of what you are responsible to report, over the past year, relative to the situation at the time of the last REB renewal (or initial approval):**

Have there been any unreported changes to the REB affecting the study documents?

No

Were there unanticipated problems, serious adverse reactions, major deviations or other events or information altering the ethical acceptability or balance between risks and benefits of the project that were not reported to the REB?

No

Were there any temporary interruptions to the project?

No

Have the results of the project been submitted for publication, presented or published?

Yes

Please specify:

1) Fierheller CT, Guitton-Sert L, Alenezi WM, Revil T, Oros KM, Gao Y, Bedard K, Arcand SL, Serruya C, Behl S, Meunier L, Fleury H, Fewings E, Subramanian DN, Nadaf J, Bruce JP, Bell R, Provencher D, Foulkes DW , El Haffaf Z, Mes-Masson A-M, Majewski J, Pugh TJ, Tischkowitz M, James PA , Campbell IG, Greenwood CMT, Ragoussis J, Masson J-Y, Tonin PN. A functionally impaired missense variant identified in French Canadian families implicates FANCI as a candidate ovarian cancer-predisposing gene. Genome Med 2021 13(1):186. (Publication attached here)

2) Wejdan M Alenezi, Caitlin T Fierheller, Timothée Revil, Corinne Serruya, Anne-Marie Mes-Masson, William D Foulkes, Diane Provencher, Zaki El Haffaf, Jiannis Ragoussis, Patricia N Tonin. Case Review: Whole-Exome Sequencing Analyses Identify Carriers of a Known Likely Pathogenic Intronic BRCA1 Variant in Ovarian Cancer Cases Clinically Negative for Pathogenic BRCA1 and BRCA2 Variants. Genes (Basel). 2022 Apr 15;13(4):697. (Publication attached in section "file upload")

3) Wejdan M. Alenezi, Larissa Milano, Caitlin T. Fierheller, Corinne Serruya, Timothée Revil , Kathleen K. Oros, Supriya Behl , Suzanna L. Arcand,
Porangana Nayar, Dan Spiegelman,Simon Gravel, Anne-Marie Mes-Masson, Diane Provencher, William D. Foulkes, Zaki El Haffaf, Guy Rouleau, Luigi Bouchard, Celia M. T. Greenwood, Jean-Yves Masson, Jiannis Ragoussis, Patricia N. Tonin. The Genetic and Molecular Analyses of RAD51C and RAD51D Identifies Rare Variants Implicated in Hereditary Ovarian Cancer from a Genetically Unique Population. Cancers (Basel). 2022 May; 14(9): 2251. (Publication attached in section "file upload")

Attach a copy of the publications, if available

FANCI.pdf

Should the REB be notified of a conflict of interest situation (of any kind) affecting one or more members of the research team, that was not reported at the time of the last approval of the project?

No

Has there been an allegation related to a breach in ethical compliance (eg: complaint from a participant, non-compliance with rules relating to ethics or integrity) concerning one or more researchers?

No

Does the sponsor require the submission of minor deviations from the protocol or other report that does not identify any impact on participant safety?

No

6. **Please attach all other documents relevant to this notification, if applicable.**

WES case review.pdf

RAD51C and RAD51D.pdf

Signature

Answer of: Tonin, Patricia

1. **I certify that the information provided on this form is correct.**

Patricia Tonin
2022-06-09 11:39

Centre universitaire de santé McGill | McGill University Health Centre

Project number(s): **MP-37-2019-4783**
Nagano identifier: **CPG-Ovarian Cancer**

Form: **F9H-96600**
Transmission date : : **2022-07-25 11:19**

File transmission report to participating centers

The REB decision regarding the notification form F9H-96600 is sent to you in this mailing. Files related to this notification are also sent to you as described below.

Files linked to this notification and transmitted by the REB :

- RAD51C and RAD51D.pdf / RAD51C and RAD51D.pdf
- WES case review.pdf / WES case review.pdf
- FANCI.pdf / FANCI.pdf

Files linked to this notification and transmitted by the REB to the answering site only :

No elements

Files linked to this notification and NOT transmitted by the REB :

No elements

The files sent with this notification can be viewed directly in the project, under the "Uploaded files" tab, in the files category called "Fichiers reliés aux suivis du CÉRÉ".

Comments (from REB evaluator) regarding transmitted files :

Appendix X: Thesis Non-Exclusive License

McGill

THESIS NON-EXCLUSIVE LICENSE

AUTHOR'S NAME: ALENEZI (*LAST NAME*) WEJDAN M (*FIRST NAME*) WMA (*INITIALS*)

AUTHOR'S DATE OF BIRTH: 12 (*MONTH*) / 26 (*DAY*) / 1982 (*YEAR*)

MCGILL EMAIL ADDRESS: wagdan.alenizy@mail.mcgill.ca **STUDENT NUMBER:** 260491470

PERMANENT ADDRESS: Medina, Saudi Arabia (*COUNTRY*)

MCGILL UNIT: Human Genetics

FACULTY: School of Biomedical Sciences

DEGREE SOUGHT: Doctor of Philosophy in Human Genetics

TITLE OF THESIS: The genetic analyses of DNA repair pathway genes in French Canadians of Quebec identified new candidate risk variants implicated in hereditary ovarian cancer

I hereby promise that I am author of the thesis above cited.

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I confirm that I have executed a **Non-exclusive license with the Library and Archives Canada** and hereby grant permission to McGill University to submit the abstract and my thesis to the **Library and Archives Canada** in full compliance with these non-exclusive licenses.

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I agree to indemnify and hold McGill University harmless against any claim and any loss, damage, settlement cost or expense (including legal fees) incurred by McGill University and arising out of, or in connection with, my statements and representations or this license.

SIGNATURE OF AUTHOR: Wejdan Alenezi Digitally signed by Wejdan Alenezi Date: 2022.12.07 21:19:17 +03'00' **EFFECTIVE DATE:** December 10, 2022

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REFERENCES

1. Brenner, D.; Poirier, A.; Smith, L.; Aziz, L.S.; Ellison, L.; Fitzgerald, N.; Saint-Jacques, N.; Turner, D.; Weir, H.K.; Woods, R.; et al. Members of the Canadian Cancer Statistics Advisory Committee Analytic Leads Additional Analysis Project Management. **2021**.
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